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UNIVERSITY OF ALBERTA

THE ROLE OF CYCLIC NUCLEOTIDES IN THE REGULATION OF INTESTINAL DIBASIC AMINO ACID TRANSPORT

BY

ROBERT L. TURNER

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF PHYSIOLOGY

EDMONTON, ALBERTA

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance a thesis entitled THE ROLE OF CYCLIC NUCLEOTIDES IN THE REGULATION OF INTESTINAL DIBASIC AMINO ACID TRANSPORT submitted by Robert L. Turner in partial fulfilment of the requirements for the degree of Master of Science

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DEDICATION

To Sally, I love you.

ABSTRACT

There have been several models proposed to explain the stimulatory action of neutral amino acids on lysine transport across the intestinal epithelium. These have included counter transport at either the brush-border or the basolateral membrane (Reiser and Christiansen, 1971; Cheeseman, 1983), undefined intracellular metabolic events (Munck, 1980), and more recently an allosteric modulation of the lysine carrier in the basolateral membrane (Lawless et al., 1987). This effect has been reinvestigated using the vascularly perfused small intestine of the frog. Lysine transport was assayed by continually perfusing the lumen of the intestine with 1 mM tritiated L-lysine and then measuring the rate of appearance of the amino acid in the vascular effluent, which was collected via a cannula in the portal vein. The addition of leucine to the vascular bed produced a dose-dependent increase in the transport of lysine across the basal pole of the epithelium. L-leucine concentrations of 0.01, 0.1, and 1.0 mM in the vascular bed produced increases in transport of 1.4-, 2.7- and 3.8-fold, respectively. Addition of IBMX (50 μM) to the intestinal perfusate shifted the leucine response curve very markedly to the left. The effect of leucine could be mimicked by the addition of 2.5 mM dibutyryl cAMP to the vascular perfusate. Forskolin, which directly stimulates cAMP production, also stimulated the vascular appearance of lysine. This effect

seemed be mediated by an action of cAMP on the transport process itself.

Cyclic AMP levels in various preparations of both frog and mammalian intestine did not increase in response to exposure to leucine. The protein kinase inhibitors HA-1004 and H-7 did not prevent stimulation of lysine transport by leucine. Thus, it seems that cAMP is not directly involved in this phenomenon. A substimulatory dose of forskolin was capable of potentiating the response of lysine transport to leucine perfusion. This, together with the potentiation of the response evoked by IBMX suggests a possible, indirect, modulatory role for cAMP in the leucine stimulation of lysine transport.

These findings are discussed in relation to the possible mechanisms by which leucine stimulates transmural lysine transport.

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LIST OF ABBREVIATIONS

BBM	microvillar or brush border membrane
BBMV	brush border membrane vesicles
BCH	2-amino-endo-bicyclo [2,2,1] heptane-2-carboxylic acid
BLM	basolateral membrane
BLMV	basolateral membrane vesicles
DAG	diacylglycerol
DbCAMP	dibutyryladenosine 3':5'-cyclic monophosphate
DDF	1,9-dideoxy forskolin
DMSO	dimethyl sulfoxide
H-7	(1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride
HA-1004	N-(2-guanidinoethyl)-5-isoquinolinesulfonamide hydrochloride
HPLC	high performance liquid chromatography
IBMX	3-isobutyl-1-methyl-xanthine
IP_3	inositol 1,4,5-triphosphate
J_{cm}	backflux from cell into mucosal solution
J_{cs}	flux from cell into the serosal compartment
J_{mc}	flux from mucosal solution into cell
J_{ms}	flux from mucosal surface to serosal surface
J_{sc}	backflux from serosal compartment into cell
J_{sm}	flux from serosal surface to mucosal surface
MeAIB	methyl-amino isobutyric acid
PKA	cAMP-dependent protein kinase
PKC	protein kinase C
PKG	cGMP-dependent protein kinase

1. INTRODUCTION

Every living creature is composed of cells. Every cell, in turn, is surrounded by a phospholipid bilayer, the plasma membrane. This 7-10 nm thick bilayer is responsible for maintaining the difference between the ambient environment and the internal composition of the cell. This difference implies that the plasma membrane is selective in what it allows to enter, or conversely to exit the cell. Indeed, this is the case. The cell membrane is virtually impermeable to many solutes such as large, polar molecules or ions (Alberts et al., 1989). A fundamental problem therefore faces the cell; how does it acquire the necessary ions and other solutes necessary for its normal functioning, maintenance and growth?

The answer comes in the form of various membrane-spanning proteins which allow specific ions or molecules to traverse the plasma membrane. Some of these proteins form aqueous pores which allow solutes of a specific size and charge to pass through them. Other proteins fall into a class called transporters which require the specific interaction of the solutes with a region of the transport protein and some sort of conformational change to bring about exposure of the solute to the opposite face of the membrane. At the simplest level, two basic types of transport proteins can be distinguished. The first are those transporters which help a substance cross the membrane in the direction of its

electrochemical gradient. Such a system is passive (i.e. requires no energy input) and is often termed facilitated diffusion. The alternate type of transporter couples some form of energy transfer with the movement of the substrate. Because energy is coupled with these transporters, they can move substrates against their electrochemical gradient and are, therefore, concentrative. This process is called active transport (Alberts et al.). This thesis deals with the transport of amino acids by intestinal epithelial cells or enterocytes. Before proceeding with the definition of the problem and the hypothesis that has been put forth, I will briefly review the structure of the intestine (i.e. histology), the classical amino acid transport systems defined in animal cells, and the various transport systems utilized by amino acids to facilitate their absorption from the lumen of the intestine into the bloodstream.

1.1. Histology of the Intestine

The intestine is a complex organ. The wall of the small intestine is made up of four concentric layers, the serosa, the muscularis, the submucosa, and the mucosa.

The mucosa, the layer most proximal to the lumen of the intestine, is composed of a number of different regions and cell types. The surface of the mucosa is thrown up into enormous numbers of villi. These are small finger-like projections, which traditionally have been implicated to

increase the surface area of the intestine available for carrying out absorptive processes. Between the bases of the villi the mucosa is invaginated to form simple tubular glands called crypts of Leiberkuhn. A simple columnar epithelium forms this mucosal surface and is made up of three major cell types; absorptive cells, goblet cells, and endocrine cells (Bloom and Fawcett, 1975).

It is the absorptive cells or enterocytes in which I am primarily interested. These cells are responsible for the absorption of dietary nutrients. The mucosal surface of the enterocyte is differentiated into large numbers of microvilli, which serve to further amplify the surface area of the mucosa exposed to the lumen. In the cores of the microvilli are bundles of actin filaments which extend downward and merge with filaments of the terminal web. These fibers, in turn, mingle with those of the tight junctions which couple the enterocytes together at their apical margins. The tight junctions form an actual physical barrier between the microvillar membrane [brush border membrane (BBM)] and the plasma membrane encompassing the remainder of the cell, the basal and lateral (basolateral) membrane (BLM). This barrier makes it virtually impossible for the components of these "two distinct" membrane regions to mix (Leeson et al., 1989)

Beneath the epithelial cell layer of the mucosa lies the lamina propria. The lamina propria consists of loose connective tissue that occupies the core of the intestinal

villi and the space between the crypts of Leiberkuhn. Below the lamina propria lies the muscularis mucosae, a thin layer of smooth muscle which is responsible for altering the surface topography of the mucosa.

The submucosa is a layer of dense connective tissue, rich in elastic fibers, and occupies the space between the muscularis mucosae and the muscularis.

The muscularis is the muscular coat of the intestine and consists of an inner circular layer and an outer longitudinal layer of smooth muscle. The muscularis is responsible for peristalsis and thus helps propel the intestinal contents along the length of the intestine.

The serosa or adventitia is the outermost layer of the gut wall and is simply a continuous sheet of simple squamous epithelium separated from the underlying mucosa by a thin loose connective tissue layer.

1.2. Amino Acid Transport Systems

The majority of amino acids are dipolar ions (or zwitterions) at physiological pH. These are often referred to as neutral amino acids. However, amino acids with more complex side chains do exist and form two other fundamental groups. Amino acids with basic side chains such as lysine or arginine carry a net positive charge at physiological pH. These amino acids are representative of the dibasic or cationic amino acids. Amino acids with acidic side chains

such as aspartate or glutamate comprise the acidic or anionic amino acids (Stryer, 1981) . I am making this distinction clear because, as will be demonstrated in the following discussion, there are many types of transport systems both in the BBM and the BLM and these systems are specific as to the group of amino acids that they will transport.

Much of the early work investigating and classifying amino acid transport systems was conducted by Christensen and his colleagues. Oxender and Christensen (1963) demonstrated that in the Ehrlich ascites tumor cell, competition for mediation across the cell membrane existed to some degree between almost all zwitterionic amino acids and that at least two different transport systems with overlapping specificities were responsible for neutral amino acid transport. They named these two classes of transport systems A and L. The A system is an Na^+-dependent system so named for its preference for alanine as a substrate whereas the L system is Na^+-independent and is leucine-preferring. A component of alanine uptake was observed to be very difficult to inhibit using a combination of methyl-amino isobutyric acid (MeAIB, a synthetic, high affinity substrate for system A) and phenylalanine. This suggested that another mediating pathway was responsible for a component of alanine flux (Christensen, 1969). Indeed, this was the case and a third neutral amino acid transport system was characterized for which typical substrates proved to be

alanine, serine, and cysteine. This Na^+-dependent mediating system was named system ASC.

In contrast, some very specific amino acid transport systems have been identified in other cell types. A transporter specific for glutamine, histidine and asparagine (system N) has been identified in rat hepatocytes and in a hepatoma cell line (Kilberg et al., 1980.; Vadgama and Christensen, 1983). In pigeon erythrocytes, rat hepatocytes and Ehrlich cells a glycine-specific system and one that recognizes β-amino acids (system β) were revealed (White, 1982).

A transfer mechanism specific for cationic amino acids was identified in the Ehrlich cell by Christensen (1964). This system was originally called system Ly^+, but the designation y^+ was invoked to avoid the implication that this system is specifically related to lysine (White, 1982). In cultured human fibroblasts, neither MeAIB or BCH (2-(-)-endoamino-bicycloheptane-2-carboxylic acid: an analog specific for system L) are capable of inhibiting lysine or arginine uptake (White, 1985). Experiments such as these support the notion that lysine or arginine uptake in these cells is not mediated by either system A or system L but by a distinct route. System y^+ has been extensively characterized and appears to be Na^+-independent, pH-insensitive and able to recognize cationic amino acids with high affinity; K_t values (the concentration of substrate that achieves half maximal transport) ranged between 0.025

and 0.2 mM (White, 1985). System y^+ can transport certain neutral amino acids in an Na^+-dependent manner and this interaction is completely abolished in the absence of Na^+ (White, 1985).

These are the classically defined amino acid transport systems (see Table 1a for a summary of these systems). By and large they have been categorized in non-epithelial cells. There are, however, a number of transport systems which do not fall into any of these aforementioned classes. Some are transporters used by epithelial cells, such as those lining the intestine.

1.3. Intestinal Absorption

Absorption is arguably the most important function of the digestive tract. This process is carried out by the enterocyte and it is generally a two-step process. Initially, amino acids enter the enterocyte by transport mechanisms located in the brush border membrane (BBM). It is this step that is usually active or concentrative. The active transport of these substrates was the focus of much investigation early on. Numerous studies investigating amino acid and sugar transport led to the development of the Na^+-gradient hypothesis. Briefly, it states that substrates such as amino acids are co-transported with Na^+ across the BBM. Na^+ moving down its electrochemical gradient into the cell drives the active transport of amino acids or

TABLE 1a

Summary of some known amino acid transport systems in tissues and cells of higher animals

System	Na^+-dependent	Typical Substrates
ASC	yes	dipolar amino acids, ubiquitous, excludes N-methylated amino acids
A	yes	dipolar amino acids, ubiquitous, N-methylated amino acids
N	yes	Gln, Asn, His
β	yes	β-amino acids
L	no	dipolar amino acids, ubiquitous, bicylic amino acids
y^+	no	cationic amino acids, ubiquitous

alternatively hexoses. The amino acid molecule then makes use of transport systems located in the basolateral membrane (BLM) to facilitate its diffusion from the cell cytoplasm into the submucosal spaces and bloodstream.

The following is a brief discussion of the various types of amino acid transport systems that have been characterized in the brush border and basolateral membranes of the enterocyte (see Table 1b for a summary of intestinal amino acid transport systems). Apparently distinct carrier systems have been described in the brush border and basolateral membranes for four classes of amino acids (A.A): α-amino-mono-carboxylic (neutral) A.A., cationic (basic) A.A., anionic (acidic) A.A., and imino acids.

1.3.1. Brush Border Transport

1.3.1.1. Neutral amino acids (mono-amino-mono-carboxylic or N-substituted amino acids):

The active nature of intestinal amino acid transport was first demonstrated by Wiseman (1953) who showed that the transport of the L-isomers of alanine, phenylalanine, methionine, histidine and isoleucine occurred against a concentration gradient. These findings were confirmed subsequently by several groups (Agar et al, 1953; Smyth and Whaler, 1953; and Wilson and Wiseman, 1954). Wiseman (1955) demonstrated that neutral amino acids compete during

TABLE 1b

Summary of some known amino acid transport systems of apical and basolateral membranes of intestine

System	Occurance	Na^+-dependent	Typical Substrates
NBB	BBM	yes	most neutral amino acids
IMINO	BBM	yes	imino acids, Pro, MeAIB
PHE	BBM	yes	Phe, Met
y^+	BBM/BLM	no	cationic amino acids, may be partially Na^+-dependent, inhibited by neutral amino acids
Proline system	BLM	no	Pro, excludes MeAIB
ASC	BLM	yes	dipolar amino acids, ubiquitous, excludes N-methylated amino acids
A	BLM	yes	dipolar amino acids, ubiquitous, N-methylated amino acids
L	BBM/BLM	no	dipolar amino acids, ubiquitous, bicylic amino acids

absorption from hamster small intestine and suggested that they were transferred by a common carrier mechanism. However, evidence that a carrier specific for imino acids such as proline or betaine argued that more than one transfer agency for neutral amino acids existed (Lin et al., 1962; Hagihira et al., 1962). Daniel et al. (1969) and Newey and Smyth (1964) suggested that more than one system for brush border neutral amino acid transport existed based on mutual competition experiments that revealed some amino acids could inhibit transport of other neutral amino acids to different extents. Indeed Newey and Smyth (1964) postulated the existence of a Pro/Gly transporter and a more general Pro/Gly/Met transporter in the rat intestine. The existence of a specific imino acid transporter was supported by the findings of Munck (1966). However, in contrast to the findings of Newey and Smyth (1964), he determined that this carrier was capable of transporting glycine, leucine and alanine in addition to betaine, proline, hydroxyproline and sarcosine. Hajjar and Curran (1970) examined the specificity of neutral amino acid transport in the rabbit ileum. At this time they favored the existence one brush border neutral amino acid transporter. The apparent affinity of amino acids for this transporter was proportional to the hydrophobicity of the side chain.

Further evidence supporting the notion of multiple neutral amino acid carriers was presented by Schultz and Markschied-Kaspi (1971). They demonstrated that the

respective K_i's (the concentration of inhibitor which produces half maximal inhibition) and K_t's for alanine and phenylalanine influx across the brush border membrane of rabbit ileum did not correspond to a strictly competitive interaction in which case the K_i and K_t should be equal. They postulated that two different carrier mechanisms are responsible for the influxes of alanine and phenylalanine. In spite of this evidence, Preston et al. (1974) state quite clearly that only one system is responsible for neutral amino acid transport. Sepulveda and Smith (1978) concluded that at least two systems exist for the mediated entry of neutral amino acids in the rabbit ileum which supports the hypothesis of Schultz and Marksheid-Kaspi (1971).

Obviously, there was much circumstantial evidence supporting the notion of multiple brush border neutral amino acid transporters. But it was not until the advent of membrane vesicle techniques that this question was resolved once and for all. Membrane vesicle techniques have many advantages over traditional *in vitro* procedures. The problems of unstirred layers (an area of reduced flow adjacent to the mucosal surface of the epithelium which represents a barrier to diffusion), and cellular metabolism are virtually abolished in vesicle preparations. Further, the transport of substrate can be examined for a specific membrane region of the enterocyte. Either brush border membrane vesicles (BBMV) or basolateral membrane vesicles (BLMV) can be prepared to a high degree of purity allowing examination of

transport characteristics specific to that membrane. Stevens et al (1982) clearly demonstrated the existence of several neutral amino acid transfer agencies in BBMV from rabbit jejunum. This was a very thorough study and used a wide range of concentrations of inhibiting amino acids. They described a system reminiscent of system L: this system was Na^+-independent and typically interacted with amino acids such as leucine, BCH (2-amino-endo-bicyclo [2,2,1] heptane-2-carboxylic acid and branched and cyclic amino acids, while it excluded β-alanine. Three Na^+-dependent pathways were disclosed as well. A system that seems to transfer most neutral amino acids yet excludes MeAIB and β-alanine was entitled the neutral brush border (NBB) system. These two major routes of neutral amino acid transfer have been confirmed in BBMV prepared from canine jejunum (Bulus et al., 1989). A proline/MeAIB pathway which transports imino acids and MeAIB (designated the IMINO carrier) which has also been reported in the mouse small intestine (Karasov et al., 1986), and a so-called PHE system which transports phenylalanine and methionine were also described. This system seems species-specific since, in the mouse, leucine and methionine inhibit each other's uptake 100 % implying that a mediator like the Phe/Met system which excludes leucine is not present (Karasov et al., 1986). Munck (1985a, 1985b) also revealed several amino acid transport pathways in the brush border membrane of rabbit ileum, each one of which had some capacity to interact with neutral

amino acids. He distinguished these pathways based upon there interaction with Na^+, affinity for neutral amino acids, and ability to interact with cationic amino acids and imino and non-α-amino acids. Numerous pathways were also revealed in the guinea pig small intestine (Satoh et al. 1989). These authors demonstrated three Na^+-dependent neutral amino acid mediators (including one in which proline and MeAIB are the principle substrates, i.e. IMINO system) and a Na^+-independent neutral amino acid transport system.

Regardless of the exact specificity of brush border neutral amino acid carriers, it should be apparent from this brief account that neutral amino acids gain access to the enterocyte cytosol via a number of different transport systems and that clearly, the influx of neutral amino acids across the brush border membrane cannot be explained by one ubiquitous transport system.

1.3.1.2. Cationic amino acids

Cationic amino acids such as lysine or arginine were shown early on to be transported against an electrochemical gradient. Hagihira et al. (1961) demonstrated active transport in the hamster intestine while Munck and Schultz (1969a) confirmed distribution ratios greater than one in sections of rabbit ileum. Findings such as these clearly indicated that the dibasic amino acids must be interacting with some form of active transport mediator.

Munck and Schultz (1969a,b) described the influx of lysine across the brush border pole of the enterocyte in rabbit ileum as the sum of two saturable processes. One is a high affinity, low capacity mechanism and the other a low affinity, high capacity mechanism. They noted that lysine influx is inhibited by replacement of Na^+ with choline. The percent inhibition by such treatment was greatest at high lysine concentrations which they interpreted as reflecting Na^+-dependency of the low affinity, high capacity carrier (which predominates at high lysine concentrations). This is in contrast to findings in rat whereh brush border lysine transport is the sum a single saturable process plus a diffusional component (Munck and Rasmussan, 1979).

1.3.1.2.1. Na^+-dependency

The question of the Na^+-dependency of brush border lysine transport is one that seems not yet to have been fully resolved. Munck and Schultz (1969a) noted that the transmural transport of lysine across rabbit ileum is reduced when Na^+ is removed from the bathing medium. Yet there was still a significant component of lysine uptake that did not seem affected by replacement of Na^+ with choline. Reiser and Christensen (1973) also observed partial Na^+-dependence of lysine uptake into isolated rat enterocytes. Paterson et al. (1981) demonstrated, quite

clearly, a Na^+-dependent portion of lysine uptake into rabbit ileum. Na^+-independent uptake of lysine was reported in brush border membrane vesicles prepared from rabbit ileum and guinea pig small intestine (Stevens et al., 1982; Satoh et al., 1989). These results were confirmed in preparations of rat BBMV (Cassano et al., 1983). Cassano et al. (1983) reported Na^+-independent, stereospecific lysine uptake. They observed no difference in uptake rates either in the presence or absence of Na^+. Most remarkable, however, was the lack of evidence for any kind of overshoot phenomenon, indicative of active transport. These results clearly contradict previous findings and also fail to answer the question of how the concentrative transport of lysine, observed in tissue preparations (Hagihira et al., 1962; Munck and Schultz, 1969a) occurs. The driving force did not appear dependent on the membrane potential or on a proton gradient, factors which have previously been identified as driving active lysine transport (Ahearn and Clay, 1987; Lee and Pritchard, 1983). Cassano et al (1983) state quite clearly that their attempts to identify a driving force for lysine transport had failed but they do suggest that these results may be due to intrinsically slow transport rates and dissipation of the putative driving force before stimulation can be observed.

In contrast, Wolfram et al. (1984) were successful in demonstrating a Na^+-dependent overshoot of lysine and arginine in BBMV from rats fed either a high-protein or a

high-carbohydrate diet. This suggests that at least a portion of brush border lysine transport is Na^+-dependent and that this might suffice to explain the concentrative uptake of lysine and arginine observed in whole tissue preparations. Interestingly, however, the uptake was not influenced by the membrane potential. Further evidence that the uptake of lysine by the brush border membrane is influenced by Na^+ comes from the work of Armstrong and his colleagues. Armstrong et al. (1988) reported a self-inhibitory action of lysine when transport is examined in Na^+-free conditions. They suggested that this represents binding of the ϵ amino group of lysine to an Na^+-binding site on the transporter which then has zero or near-zero mobility. If this is the case, then it is obvious that the presence of Na^+ is important for efficient transfer of lysine across this membrane.

The evidence is confusing. But one can conclude that the transport of lysine is partially dependent on Na^+. This, of course, suggests that lysine transport is achieved, in part, via a transporter that couples lysine and Na^+ moving down its concentration gradient. Clearly, there is also a portion of lysine transport that does not require Na^+ for activation.

1.3.1.2.2. Interaction with neutral amino acids

There is much evidence to suggest that the brush border transporter of cationic amino acids has overlapping specificity with neutral amino acids.

This phenomenon was probably first observed by Munck and Schultz (1969b). They found that lysine was capable of inhibiting the brush border flux of leucine and that leucine was capable of doing the same to lysine flux. Preston et al. (1974) notes that cationic amino acids could reduce that influx of methionine into the enterocyte by 30 %. A similar reciprocal pattern of inhibition as was observed by Munck and Schultz (1969b), was observed between alanine and lysine (Paterson et al., 1981). BBMV's prepared from guinea pig small intestine demonstrated the ability of lysine to inhibit the Na^+-independent uptake of leucine, phenylalanine, and cysteine and the corresponding ability of each of these neutral amino acids to inhibit the uptake of lysine. Lysine apparently had no effect on the Na^+-dependent uptake of a series of neutral amino acids (Satoh et al., 1989). It appears that the mouse intestine has a single shared Na^+-independent amino acid carrier (i.e. is capable of transporting lysine, leucine and methionine) which excludes imino acids and acidic amino acids (Karasov et al., 1984). Karasov et al. (1986) also report that in the mouse the uptake of lysine is completely blocked by leucine, while lysine only inhibits leucine transport by

approximately 40%. This suggests that as in rabbit, rat and guinea pig, neutral amino acids have access to the basic amino acid carrier (Karasov et al., 1986).

1.3.1.3. Anionic amino acids

Early attempts to demonstrate transport of anionic amino acids (L-glutamate, L-aspartate) against a concentration gradient were not successful. Both *in vivo* and *in vitro* methods failed to demonstrate intracellular or serosal fluid concentration of L-glutamate or L-aspartate (Wiseman, 1953; Spencer et al., 1966; Lerner and Steinke, 1977; Lin et al., 1962)

There was no shortage, however, of evidence suggesting that some sort of specific transport systems for the anionic amino acids exists. Gibson and Wiseman (1951) observed that the L-isomer of glutamate is absorbed more rapidly, *in vitro*, than the D-isomer. The transepithelial potential difference (lumen negative) was shown to increase in magnitude, as measured by the short circuit current, upon the addition of L-glutamate to the mucosal solution (Schultz and Zalusky, 1965). Schultz et al. (1970) measured uptake of anionic amino acids using short incubation times and without the influence of tissue metabolism. They found that uptake was saturable, subject to competitive inhibition and was influenced by the Na^+ concentration. The sensitivity to the Na^+ concentration suggests that the mechanism of uptake

observed for the other amino acids (i.e. Na^+-coupled transport) may be operating in the case of the anionic amino acids. Indeed it has been postulated that the failure to demonstrate transport of these amino acids against a concentration gradient is because of the rapid transamination that these amino acids undergo upon entry into the enterocyte (Neame and Wiseman, 1957). This argument is further strengthened by the observation that glutamate uptake is inhibited by metabolic poisons, and Na^+ replacement (Lerner and Steinke, 1977).

Again, due to the advent of membrane vesicle techniques which overcome the problem of metabolism, it was possible to demonstrate a stimulatory effect of a Na^+ gradient on both glutamate and aspartate transport (Corcelli et al., 1982). As well, an overshoot phenomenon was observed indicating, clearly for the first time, the active nature of this transport system. Saturation of the transport system and mutual inhibition between glutamate and aspartate was also demonstrated indicating that the same transporter serves both amino acids. It appeared that the system was non-electrogenic since imposition of a valinomycin-induced K^+ diffusion potential (inside negative) did not alter the transport rates. This would be expected if the ratio of Na^+-to-glutamate or aspartate is one-to-one or if an anion (i.e. Cl^-) was also involved in some form of counter-exchange.

Thus it seems that anionic amino acids cross the brush border membrane largely via a separate carrier specific for glutamate and aspartate which appears to be Na^+-dependent. The picture may be a little more complicated than this, however. Corcelli and Storelli (1983) recently demonstrated that intravesicular K^+ and extra- and possibly intravesicular Cl^- was necessary for maximum activation of the Na^+/acidic amino acid cotransporter. They suggest that K^+ and Cl^- are not translocated across the membrane but rather are required for maximal activation of the Na^+/acidic amino acid cotransport system.

1.3.2. Basolateral Transport

The transepithelial transfer of amino acids from the lumen of the intestine to the plasma requires transport across the basolateral membrane. Initially, investigations of amino acid transport demonstrated that the cumulative element of transport occurred at the level of the brush border membrane (Akedo and Christensen, 1962; Kinter and Wilson, 1965). Early work with membrane vesicles demonstrated conclusively that indeed, it is the brush border transport systems that are capable of active amino acid transport (Hopfer et al., 1976). Results of experiments using indirect methods have suggested the presence of Na^+-independent facilitated diffusion mechanisms in the basolateral membrane (Munck, 1965; Hajjar et al.,

1972; Bihler and Cybulsky, 1973). Hopfer et al. (1976) directly established the existence of a Na^+-independent neutral amino acid transport system in the basolateral membrane.

Since this original work, not surprisingly, a number of different amino acid transport systems in the basolateral membrane have been characterized. The following discussion will elaborate on the various transport systems found in this membrane region.

1.3.2.1. Neutral amino acids

As mentioned previously, Hopfer et al. (1967) demonstrated saturable, Na^+-independent valine transport in basolateral membrane vesicles. They did not conduct experiments examining the specificity of the basolateral transport pathways. Consequently, much of our knowledge concerning neutral amino acid transport across the basolateral membrane comes from the work of Wright and his colleagues. Mircheff et al. (1980) examined in detail the basolateral transport pathways for alanine and examined the inhibitory effect of a number of neutral amino acids. Four separate alanine transfer agencies were determined, one of which was Na^+-independent. Of the other three Na^+-dependent systems, one exhibited specificity of the A-type and another exhibited specificity reminiscent of system ASC. The third system seemed novel in that it was inhibited by MeAIB but

not by phenylalanine. Mircheff et al. (1980) postulated that the "L-like" system was responsible for the exit step in the overall process of neutral amino acid active absorption. These findings have been confirmed in subsequent investigations (Cheeseman, 1981; Ghishan et al., 1989).

Another carrier capable of transporting neutral amino acids was uncovered by Davies et al. (1987). They demonstrated the presence of a carrier in which L-proline appears to be the primary substrate. Leucine, alanine, and glycine appear to be able to inhibit this system and thus probably can be transported by this system as well.

1.3.2.2. Cationic amino acids

The slow transmural transport rate for the dibasic amino acids has been attributed to the transport process located in the basolateral membrane (Munck and Schultz, 1969a, 1969b; Cheeseman et al., 1983).

Cheeseman (1983) demonstrated clearly, for the first time, the existence of a transport system in the intestinal basolateral membrane specific for dibasic amino acids. Ornithine, arginine, and lysine appeared to share a common transport system.

A more complete study of the characteristics of basolateral lysine transport was carried out using rat jejunal BLMV's (Lawless et al., 1987). Competition

experiments indicated that this carrier was specific for ornithine, lysine, arginine, and histidine. This specificity and its lack of Na^+-dependence places this basolateral carrier in the class designated y^+. Kinetic analysis of the data indicated that the maximal rate of transport (V_{max}) was similar to other amino acid transport systems in this membrane. Yet the observed K_t was an order of magnitude larger (33 mM as opposed to the non-epithelial y^+ K_t of 0.025 to 0.2 mM). It was postulated that this low affinity for lysine might explain the relatively slow rate of transport of this amino acid across the basolateral membrane.

1.3.2.3. Anionic amino acids

The transport of glutamate and aspartate across the basolateral membrane has not been investigated thoroughly. This is probably due in large part to the assumption that these amino acids are rapidly and effectively transaminated to other amino acids such as alanine by the intestinal epithelia (Neame and Wiseman, 1957). This is assuming that relatively low concentrations of these amino acids are present in the intestinal lumen. But as Neame and Wiseman (1957) have shown, when higher concentrations of either aspartate or glutamate are presented to the lumen, there is a marked increase in the levels of these substrates in the venules draining the intestine. This may imply that

aspartate and glutamate are transported across the basolateral membrane via some carrier mechanism. Himukai (1984) has demonstrated similar findings with everted sacs of guinea pig intestine (i.e. increased appearance of glutamate in the serosal bathing medium when the mucosal surface in immersed in a glutamate medium). Membrane vesicles techniques have demonstrated that, by and large, acidic amino acid transport across this membrane is Na^+-dependent and is hypothesized to mediate uptake by the enterocyte of these amino acids from the bloodstream (Reshkin et al., 1988). However, Scalera et al. (1984) demonstrated both an Na^+-dependent and a Na^+-independent uptake pathway for aspartate and glutamate. They suggest that during intestinal absorption, acidic amino acids are concentrated in the mucosal cells and then released into the serosal compartment via the Na^+-independent mediating mechanism. However, membrane vesicle techniques have not yet been used in depth to investigate the possibility of carrier-mediated transport of dicarboxylic amino acids across the basolateral membrane, and so to a large extent, our knowledge about this process is incomplete.

1.4. The lysine-leucine phenomenon

1.4.1. Background

The ability of certain neutral amino acids such as leucine and methionine to stimulate the intestinal transport of dibasic amino acids like lysine has been known for over twenty years and has been demonstrated in a number of animal species (Munck and Schultz, 1969a,b; Cheeseman, 1983; Munck, 1989). This phenomenon was first observed by Robinson and Felber (1964). They found that when sections of rat intestine are exposed to neutral amino acids such as L-leucine, L-methionine, or L-citrulline, their ability to take up the dibasic amino acids L-arginine and L-lysine is increased. Lysine or arginine uptake could be stimulated 1.2- and 2- fold, respectively. However, Robinson and Felber (1964) did not propose a mechanism for this stimulation and little physiological significance was placed on these observations.

Munck (1965) noted a marked enhancement of the transepithelial transport of L-lysine in everted intestinal sacs preloaded with leucine or when the mucosal surface of the intestine was exposed to both leucine and lysine together. Transepithelial transport was stimulated approximately three-fold. This effect was explained as an example of a counterflow phenomenon which must be acting on either the serosal surface of the basolateral membrane or

the intracellular surface of the luminal part of the plasma membrane. The counterflow phenomenon or *trans*-stimulation is a phenomenon observed when two compounds (i.e. substate A and B for the purposes of this explanation) share the same transporter. The presence of substrate B on the opposite, or *trans*-side, of substrate A will increase the rate at which substrate A is translocated because substrate B will use the same transporter and move in the opposite direction of A. This will decrease the time necessary for the transporter to cycle back to the face of the membrane where substrate A is located and thereby increase the overall rate of substate A transport.

Work conducted on Ehrlich ascites tumor cells demonstrated that the accumulation of tryptophan in these cells could be augmented by the presence of neutral amino acids (Jaquez, 1963). It was suggested that this stimulation was due to the presence of one type of carrier (i.e. for both amino acids) in which both binding sites must be occupied before the carrier can effect transport. Munck (1966) set out to provide evidence that the counterflow phenomenon is presiding in the case of intestinal dibasic amino acid transport. He assumed that the carriers for neutral and dibasic amino acids functioned according to the concepts of Wilbrandt and Rosenberg (1961). Basically, he demonstrated that conditions for a counterflow situation are present and can explain the data collected to this point in time, adequately. In this situation, a counterflow

phenomenon requires that there be separate transporters for neutral and cationic amino acids. Munck (1966) states that this is true based on the small degree of competition exhibited between the amino acids of these two groups. Further, the K_t of lysine for the cationic carrier and the K_t of lysine for the neutral carrier should be small and large, respectively. The K_t of lysine for the cationic carrier was determined by Munck (1966) to be 0.3 μM. This estimate is subject to error since it was arrived at by measuring the concentration-dependence of lysine flux into intestinal sacs. Thus it was determined by measuring the transmural flux which is dependent on two transport systems, one in the brush border membrane and a second in the basolateral membrane. The high K_t of lysine for the transporter of neutral amino acids is demonstrated by the inability of lysine to inhibit the flux of leucine. Munck (1966) also states that neutral amino acids like leucine should have some affinity for the cationic amino acid carrier. This is demonstrated by the ability of 15 mM leucine to inhibit the transport of lysine by up to 40 %. Finally, if leucine is to drive the counterflow of lysine, then there should be a accumulation of leucine on the serosal side of the transport systems as is demonstrated by the uphill transport of leucine into the serosal compartment of everted sacs (Munck, 1966). The model proposed by Jaquez (1963) to explain the stimulation of tryptophan transport in Ehrlich ascites tumor cells by methionine is not valid in this situation due to

the persistence of the leucine stimulatory effect at high concentrations of lysine (Munck 1966). This fact, plus evidence in support of conditions favorable for counter-transport, led Munck (1966) to conclude that a counter-transport phenomenon was responsible for the neutral amino acid stimulation of lysine transport.

Up to this point, however, no investigator had attempted to determine at which membrane of the intestinal epithelial cell (or enterocyte) the stimulatory action of leucine on lysine transport was occurring. The plasma membrane of the enterocyte is separated by tight junctions into two functionally distinct membrane units, the brush border membrane and the basolateral membrane. It is possible that leucine could be affecting the dibasic amino acid carriers at either of these membranes or at both. In an attempt to answer this question, Munck and Schultz (1969a) investigated interactions between leucine and lysine transport employing techniques that allow indirect calculations of amino acid fluxes at both of these functionally distinct membranes.

The transport of an amino acid from the lumen of the intestine to the bloodstream requires, minimally, two transport steps. First the amino acid must be transported from the bulk phase in the intestine across the brush border and into the cytoplasm of the enterocyte. From the enterocyte the amino acid is transported across the basolateral membrane into the submucosal layers of the tissue from where it diffuses into the blood. Clearly then,

net transmural transport is dependent on at least four factors: these are i) the rate of transport from the mucosal solution into the cell (J_{mc}), ii) the resulting backflux from the cell into the mucosal solution (J_{cm}), iii) the rate of transport from the cell into the serosal compartment (J_{cs}), and iv) the resulting backflux from the serosal compartment into the cell (J_{sc})[1]. It is difficult to determine the magnitude of all these fluxes directly. However, it is possible to determine some of them. The transmural transport rate is easily determined and is dependent upon the flux from mucosa to serosa (J_{ms}) and the backflux from serosa to mucosa (J_{sm}). Thus net transport from the intestinal lumen to the serosal side is equal to $J_{ms}-J_{sm}$. The flux of amino acids into the cell from the intestinal lumen is likewise easily determined enabling the determination of J_{mc}.

Munck and Schultz (1969a) observed a four fold increase in the rate of net transmural lysine transport when leucine was present in the mucosal incubation medium. They established that this was due solely to an increase in J_{ms} and that J_{sm} was not affected. In the steady state, an increase in net transmural lysine transport must be associated with parallel increases in transport across both the mucosal and serosal membranes of the enterocyte. An

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1. All abbreviations for fluxes refer to lysine transport unless otherwise indicated

increase in the transport of lysine into the cell could be achieved either by increasing J_{mc} or decreasing J_{cm}. Likewise the required increase in transport across the serosal membrane could be achieved by increasing J_{cs} or decreasing J_{sc}. Munck and Schultz (1969a) showed that in the presence of 2 mM leucine, J_{mc} from a solution of 10 mM lysine does not increase and high intracellular levels of lysine are not achieved; if anything, there is a decrease in the steady-state intracellular lysine concentration. Consequently, the increase in transport across the brush border membrane due to the presence of leucine must be due to a decrease in J_{cm}. J_{sc} is unaffected by leucine and therefore, the increase in transport across the basolateral membrane must be due to an increase in J_{cs}.

This view is supported by evidence suggesting the transport of lysine across the BLM is rate-limiting (Munck and Schultz, 1969b; Cheeseman et al., 1983). The increase in J_{cs} is very marked and required explanation. As Munck and Schultz (1969a) suggest the possibility arises that intracellular lysine is compartmentalized so that the observed fall in intracellular lysine concentration in the presence of leucine represents a shift of lysine to a "lysine transport pool" which, in turn, could result in an increased J_{cs}. Munck and Schultz (1969a), however, feel that perhaps intracellular leucine in some way changes the processes which mediate lysine efflux out of the cell. They support this argument by citing experimental evidence which

argues against the counter-transport hypothesis. Pre-loading the tissue with leucine does stimulate lysine transport but they suggest it cannot be attributed to a heteroexchange phenomenon because lysine homoexchange is not observed (Munck and Schultz, 1969a;b). Also the stimulation of lysine transport appears highly specific. Leucine and methionine can stimulate lysine transport whereas, in this preparation, the vast majority of other neutral amino acids appear unable to stimulate transport. Munck and Schultz (1969a) suggest that intracellular leucine may interact non-competitively with the lysine transport mechanism in the BLM and result in configurational changes that increase the rate at which the lysine-carrier complex translocates across this membrane.

Reiser and Christiansen (1971) investigated the "leucine effect" further in everted intestinal sacs and isolated enterocytes. With regard to experiments with everted sacs, their results are in agreement with those of Munck and Schultz (1969a). They found that leucine, methionine, and alanine could increase the net transmural lysine transport to a greater or lesser extent. The neutral amino acids valine and isoleucine did not seem to affect lysine transport. In each case, as was determined by Munck and Schultz (1969a), such stimulation of lysine transport did not seem to be associated with an increase in the intracellular concentration of lysine as would be expected if brush border lysine uptake was being stimulated. Indeed,

when the time-course of the leucine effect was followed, it was found that the serosal appearance of lysine was significantly stimulated at incubation times as short as 5 minutes without a parallel change in intracellular lysine concentration. As pointed out by Reiser and Christiansen (1971), if we assume that during this first 5 minutes, it is primarily the mucosal to cell flux of lysine under examination, we can conclude that the stimulation of lysine transport by leucine is not achieved by increasing this parameter (J_{mc}) as is concluded by Munck and Schultz (1969a). However, they found that when sacs are preincubated in leucine, the lysine transport rate was increased; yet, in contrast to the previous condition, there was an increase in the intracellular lysine concentration. Reiser and Christiansen (1971), therefore, decided to investigate the stimulation of lysine transport by leucine in isolated intestinal epithelial cells. They assumed that this preparation would enable a more clear understanding of the interactions between neutral amino acids like leucine with lysine.

They demonstrated that the isolated enterocytes can actively accumulate lysine with medium/cell distribution ratios of 3 or more. With certain neutral amino acids (L-alanine, leucine, phenylalanine, methionine) present in the incubation medium, they noted up to a 2-fold increase in lysine uptake into the cells. Reiser and Christiansen (1971) correctly note that an increase in the steady state

level of lysine in the cell could be achieved by increasing the flux into the cells (J_{mc}) or decreasing the efflux out of the cells (J_{cm}). To distinguish between these two possibilities, they investigated the stimulatory action of neutral amino acids on lysine uptake for time intervals as short as 1 minute in the hope of measuring initial rates of transport. Indeed, they provide evidence that lysine uptake does increase after incubation in the presence of leucine for a period as short as 1 minute. This is in contrast Munck and Schultz's (1969a) work on sacs. Cells, preincubated for 10 minutes with leucine, in which lysine transport was assayed in medium devoid of leucine, maintained high rates of lysine uptake; Reiser and Christiansen (1971) state that this provides evidence that only intracellular leucine is necessary for the stimulation of lysine transport. The fact that dibasic amino acid transport can be stimulated by neutral amino acids in isolated enterocytes, Reiser and Christiansen (1971) state, is evidence against the mechanism of this interaction proposed by Munck and Schultz (1969a). Reiser and Christiansen (1971) favor exchange or counter-transport as the mechanism by which neutral amino acids stimulate the transport of dibasic amino acids. They propose that two independent transport systems play a role in this interaction. One, that of neutral amino acids, is Na^+-dependent and results in the accumulation of neutral amino acids like leucine, intracellularly. The other, that of

cationic amino acids like lysine or arginine, involves a hetero-exchange between intracellular leucine and extracellular lysine; of course, this would result in an increased net uptake of lysine and a concomitant decrease in leucine uptake. The evidence on which they base their conclusions is as follows: i). active accumulation of leucine occurs within 1 min at which time the stimulation of lysine uptake was also noted; ii) the increase in lysine uptake and decrease in leucine uptake showed similar time courses; iii) leucine appears to increase lysine uptake by increasing influx rather than efflux; iv) lysine uptake was optimally stimulated by intracellular leucine; v) both lysine and leucine displayed a tendency to participate in exchange transport.

Munck (1980) set out to investigate further the relationship between neutral amino acids and basic amino acids. Specifically, his experiments were designed to further elucidate the apparent contradictory results which demonstrate both inhibitory and stimulatory effects of neutral amino acids on the transport of dibasic amino acids such as lysine. It was found that all of the neutral amino acids tested were competitive *cis*-inhibitors (i.e. inhibited transport when the inhibitor was placed, experimentally, on the same side of the membrane as the substrate) of J_{mc} and were also *trans*-stimulators of J_{mc} with the exception of isoleucine. The *trans*-stimulatory effect also seems to be Na^+-dependent and does not correlate closely with the

effectiveness of the neutral amino acids as *cis*-inhibitors. This, plus the fact that galactose undergoes *trans*-stimulation by alanine, supports Munck's hypothesis that the *trans*-stimulation of basic amino acid uptake into epithelial cells (J_{mc}) is due to a rather non-specific hyperpolarization of the brush border membrane as a result of Na^+-coupled amino acid efflux out of the enterocyte. Munck (1980) demonstrated that the *trans*-stimulation of J_{mc} is unrelated to the stimulation of J_{ms}. He demonstrated a 60% increase in J_{ms} from a solution containing 10 mM lysine while the mucosa-to-cell flux, Jmc is in fact reduced by 50%. The neutral amino acids can be divided into two groups based on their effect on the intracellular concentration of lysine ($[Lys]_c$) and J_{ms}. Of the neutral amino acids tested, only valine, phenylalanine and α-amino-n-butyric acid (ABA) did not significantly increase J_{ms}. ABA does significantly increase $[Lys]_c$, while it appears that those amino acids that increase J_{ms} do not increase $[Lys]_c$ and, if anything, decrease it.

Munck (1980) suggests that the increase in $[Lys]_c$ when exposed to ABA or alanine may be as a result of competitive inhibition of lysine efflux from the enterocyte by the neutral amino acids. Possibly leucine has a similar effect which is overshadowed by a parallel enhancing effect across the basolateral membrane.

So, at this point we have nearly gone full circle. Munck (1965) initially speculated that a counterflow phenomenon

explained the stimulation of dibasic amino acid transport and yet he did not address the question of which membrane of the enterocyte is involved (i.e. the brush border membrane or the basolateral membrane). Later evidence suggested that transport across the basolateral membrane may be mediating the increase in transmural lysine transport and that it probably did not involve a counter-exchange mechanism (Munck and Schultz, 1969a). Munck and Schultz (1969a) suggested that leucine may be inducing some sort of configurational change in the lysine transport mechanism which increased the rate at which the lysine-carrier complex translocates across the basolateral membrane. Reiser and Christiansen (1971) made use of isolated enterocytes to delineate the leucine phenomenon. Indeed, Reiser and Christiansen's (1971) conclusion that leucine stimulates lysine transport via a counter-exchange phenomenon at the brush border is contradictory to results obtained by Munck (1980) who showed that in the steady state leucine did not stimulate J_{mc}, but, in fact, reduced this parameter. Munck (1980) maintains that the stimulation of dibasic amino acid transport by neutral amino acids is via stimulation of J_{cs} by some hitherto unknown mechanism.

It seems logical to conclude that stimulation of two processes has been observed and this dual stimulation has confused the accurate delineation of the mechanism of the leucine stimulation of lysine transport. *Trans*-stimulation of J_{mc} by leucine has been observed but this does not

explain the increase in J_{ms} because this phenomenon is not dependent on stimulation of J_{mc}. Also it appears that J_{cs} is the rate-limiting step in transmural lysine transport (Cheeseman et al., 1983; Munck and Schultz, 1969a,b). If we assume that the basolateral lysine carrier is already saturated in experimental or physiological conditions, then increasing J_{mc} would be incapable of accounting for the increase in J_{ms}.

In an attempt to reconcile these disparate data, Cheeseman (1983) investigated the transport of lysine in the vascularly perfused frog small intestine. Cheeseman (1983) demonstrated the existence of a dibasic amino acid transporter in the basolateral membrane. Furthermore, the presence of several neutral amino acids in either the lumenal or serosal perfusate stimulated the washout of lysine into the vascular bed from lysine-preloaded intestine. This is contrary to the findings of Munck and Schultz (1969a). They demonstrated that leucine was incapable of altering the rate of lysine transport when bathing the serosal side of the tissue. Nevertheless, Cheeseman's (1983) work indicates that certain neutral amino acids such as leucine do, in fact, stimulate the exit step of transmural lysine transport (J_{cs}). He proposed that this may be achieved via a hetero-counter-exchange mechanism operating at the basolateral membrane of the enterocyte.

To further characterize lysine transport across the intestinal basolateral membrane, Lawless et al, (1987)

employed membrane vesicle techniques. Several conclusions were drawn from this study. It appears that lysine transport across this membrane is unaffected by the presence of Na^+ in the incubation medium. It was also determined that the transport of lysine is associated with the net movement of positive charge since voltage-clamping the membrane potential with valinomycin did increase lysine influx into the vesicles. The V_{max} of this transport system appears quantitatively similar to other transport systems located in this membrane while the K_t is an order of magnitude larger, indicating that this may be why the *in vitro* transport of lysine across this membrane is slow when compared to other amino acids. The effect of leucine on this preparation was to stimulate transport and could be achieved with concentrations as low as 0.1 μM. This argues against counter-transport since such a situation would be thermodynamically impossible. This observation led Lawless et al. (1987) to suggest that leucine may be interacting allosterically with the transporter to somehow increase the rate of lysine transport. Further, they speculated that this leucine effect is a regulatory mechanism to promote the absorption of essential amino acids like lysine during the course of a protein-containing meal and prevent the backflux of these amino acids between meals.

1.5. Second messengers and transport

1.5.1. Second messenger systems - defined

Hydrophilic signaling molecules such as protein hormones, growth factors, and neurotransmitters bind to specific receptor proteins on the surface of the plasma membrane of target cells. Two major signal pathways are well defined. One involves the generation of cyclic adenosine monophosphate (cAMP). The other pathway generates two second messengers, inositol triphosphate (IP3) and diacylglycerol (DAG). Both these systems have been confirmed in the enterocyte (deJong, 1975; deJong, 1976; deJong and Vaandrager, 1987). The following is a brief discussion of these two major signal pathways and the sequence of events from extracellular stimulation to physiological response.

1.5.1.1. Cyclic AMP system

Sutherland and his colleaques developed the original idea of cAMP as a second messenger and elucidated the primary sequence of events in the cAMP cascade (Sutherland, 1972) (see Figure 1). Cyclic AMP is synthesized from ATP by the plasma membrane-bound enzyme, adenylate cyclase, in response to agonist-receptor interaction and is rapidly and continuously destroyed by one or more cAMP phosphodiesterases which hydrolyze cAMP to adenosine 5'-

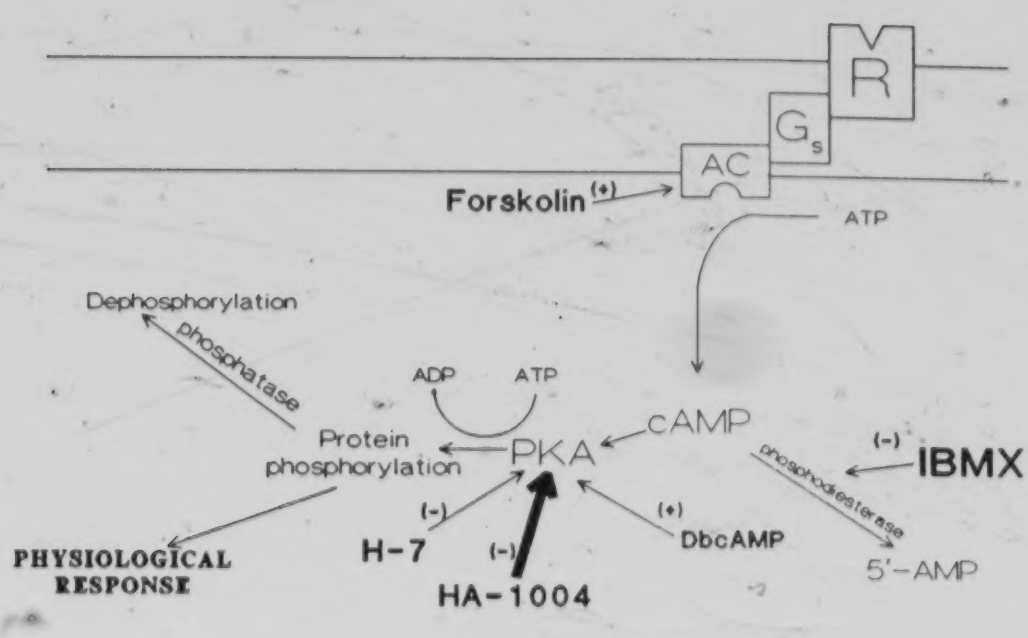

Figure 1. Cyclic AMP signal pathway. Signals from stimulatory receptor, **R**, activate adenylate cyclase (**AC**) to generate cAMP from ATP. The G-protein, G_s, governs this reaction. cAMP binds to a regulatory component of cAMP-dependent protein kinase (**PKA**), liberating the catalytic component which then can phosphorylate specific proteins that regulate a cellular response. Shown in bold are the pharmacological agents which can affect this system and their respective actions ((-)=inhibitory, (+)=stimulatory) at specific steps of this pathway.

monophosphate (5'-AMP) (Sutherland, 1972; Weishaar, 1987). Cyclic AMP, in turn, mediates the intracellular events that result in the appropriate biological response. In the 30 or so years since Sutherland's original hypothesis, many of the details of the mechanism of cAMP generation and action have been worked out. After agonist-receptor interaction, the receptor is capable of communicating with a pair of guanine-nucleotide-binding regulatory proteins (Northup et al., 1983). One, G_s, mediates stimulation of adenylate cyclase activity, while the other, G_i, is responsible for inhibition. The G-proteins are made up of at least three subunits, α, β and γ. The β and γ subunits appear identical while the α subunit seems to bear the characteristic properties of the "activated" G-protein (Northup et al., 1983). The role of the agonist-bound receptor is to facilitate the replacement of GDP by GTP on the α subunit of G_s which then allows activation of adenylate cyclase and generation of cAMP from ATP (Londos et al., 1974; Sevilla et al., 1976; Cassel and Selinger, 1976). The final chemical steps are mediated by a cAMP-dependent protein kinase (PKA). Cyclic AMP binds to PKA, dissociating this enzyme into subunits and thereby releasing an active catalytic unit. The catalytic unit is capable of phosphorylating the target protein, thus altering its activity (Gill and Garren, 1970; Stadel and Lefkowitz, 1989).

1.5.1.2. Inositol lipid system

This thesis concentrates on the cyclic nucleotide-generating system. As such, I will not discuss in too much detail the IP_3/DAG second messenger system. For two excellent reviews of IP_3 and DAG, respectively, I direct you to Berridge and Irvine (1984) and Bell (1986).

In response to a transmembrane signal (i.e. agonist binding to an α_1 receptor), phosphatidylinositol bisphosphate (PIP_2) is hydrolyzed via a GTP (G-protein)-dependent activation of phopholipase C (see Figure 2). This produces two second messengers, IP3 and DAG. DAG greatly increases the affinity of protein kinase C (PKC) for Ca^{2+} and phosphatidylserine (PS), thereby causing activation of PKC which like PKA, phosphorylates target proteins. The IP_3 which is generated functions primarily to increase intracellular Ca^{2+}. IP_3 achieves this by acting on a Ca^{2+} channel in a IP_3-sensitive pool of endoplasmic reticulum which then allows stored Ca^{2+} to be released into the cell cytosol. Ca^{2+}-binding proteins, such as calmodulin, then become saturated with Ca^{2+} resulting in a conformational change which allows the Ca^{2+}/calmodulin complex to interact with target proteins such as Ca^{2+}/calmodulin-dependent protein kinases (Ca^{2+}-kinases) which phosphorylate serine and threonine residues on proteins.

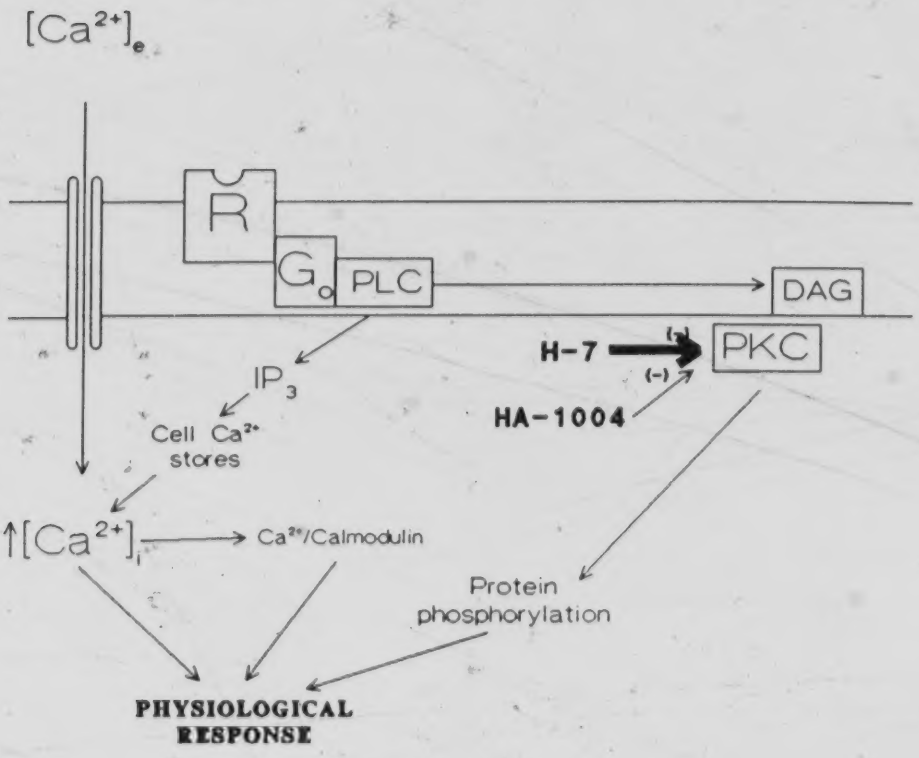

Figure 2. Inositol-lipid signal pathway. External signals bind to receptor **R**, which transmits information through a G-protein, G_o, to activate phospholipase C (**PLC**). PLC cleaves phosphatidyl inositol 4,5-bisphosphate (**PIP_2**) into the second messengers inositol triphosphate (**IP_3**) and diacylglycerol (**DAG**). IP_3 in the cytoplasm stimulates the release of Ca^{2+} from intracellular calcium stores which can stimulate a protein kinase (calmodulin). DAG remains in the membrane where it aids the the activation and translocation of protein kinase C (**PKC**) to the membrane leading to the phosphorylation of a distinct set of proteins.

1.5.2. Second messengers and the "Lysine-Leucine phenomenon"

The hypothesis that second messengers may be involved in the leucine stimulation of lysine transport was brought about by several pieces of evidence. In work conducted on rat jejunal basolateral membrane vesicles (BLMV), the degree of stimulation of lysine transport by leucine seen in more conventional *in vitro* techniques and in the vascularly perfused frog small intestine cannot be attained (Cheesman, 1983; Lawless et al., 1987). Further, the reproducibility of the effect in BLMV's was extremely variable (i.e. sometimes stimulation would occur while at other times it would not). These observations led to the idea that perhaps some soluble component is involved in the stimulation of dibasic amino acid transport by certain neutral amino acids.

1.5.3. Modulation of transport systems by second messengers

The involvement of second messengers in the regulation of transport systems is well documented. Cyclic AMP has been shown to increase the transport of amino acids in a number of tissues. Griffin and Szego (1968) demonstrated the ability of cAMP and dibutyryl cAMP (DbcAMP) to enhance the accumulation of L-leucine and lysine by segments of uterine tissue. Further, upon exposure of uterine segments to

estradiol there was an increase in leucine accumulation and cAMP content of the tissue. In rat liver, cycloleucine and 2-aminoisobutyric acid (AIB) transport is increased following administration of cAMP (Mallette et al., 1969; Chambers et al., 1970). Further support for this came from the work of Scott et al. (1970) showing that theophylline (which like IBMX is a methyl xanthine and inhibits phosphodiesterase activity) also caused an elevation in liver AIB transport. Transport of AIB was found not to increase in diaphragmatic tissue exposed to DbcAMP (Chambaut et al., 1969), while in rabbit atrial tissue, DbcAMP decreased AIB transport (Hait et al., 1972). However, in rat calvaria (bone), DbcAMP increased AIB uptake and also increased the distribution ratio of glycine and proline (Phang et al, 1970). DbcAMP increases transport of tryptophan in brain slices with a corresponding increase in serotonin synthesis (Forn et al., 1972).

There is also evidence for a direct involvement of cyclic nucleotides with substrate transport by the intestine. Kinzie et al. (1973) noted that both neutral and basic amino acid transport in rat intestine was stimulated by cAMP, DbcAMP, theophylline, and cholera toxin. Kinetically, it appears that this stimulation is mediated by a decrease in K_t. The enhanced uptake correlated with increasing tissue cAMP levels but not with cGMP levels. They conclude that these effects are through mediation of transport processes at the brush border membrane. The same workers determined

that this is a specific effect since, in their preparation, neither the uptake of imino-amino acids nor 3-O-methylglucose was affected. Burrill et al. (1974) observed a similar phenomenon with respect to methionine transport by chicken intestine. In the absence of Na^+, methionine transport proceeds with reduced affinity and maximal flux. Replacement of Na^+ restored the K_t but had little effect on V_{max}. However, in Na^+-free conditions the presence of theophylline maintained V_{max} while the K_t increased. The authors envisage a scenario wherein not only does Na^+ increase the affinity of the carrier of neutral amino acids for methionine but they propose the Na^+ is also required to maintain the activity of an Na^+-dependent protein kinase which, when activated through cAMP, enhances carrier translocation (V_{max}). In contrast to the aforementioned findings of Kinzie et al. (1973), Reymann et al. (1985) found that forskolin was able to stimulate active jejunal glucose transport *in vitro*. This was correlated with an increase in tissue cAMP levels.

Not surprisingly, cAMP is not the only second messenger capable of regulating transport systems; other second messenger systems have been implicated. For instance, protein kinase C (PKC) inhibits $Na^+/K^+/Cl^-$ cotransport in BALB/c 3T3 cells (O'Brien et al., 1988). Phorbol esters and diacylglycerol (DAG) analogues increase Na^+-dependent amino acid transport 2 to 3 fold in cultures of LLC-PK1 pig kidney epithelial cells (Dawson and Cook., 1988). This effect is

attributed to a phosphorylation of either a regulatory protein or the transporter itself.

It is evident from this brief account that second messengers can indeed influence various transport systems. Therefore, the possiblity of second messenger involvement in the leucine stimulation of lysine transport is, I feel, realistic proposition. Bearing this in mind, it was the goal of this project to reinvestigate lysine transport in the vascularly perfused frog preparation to elucidate the possible involvement of second messengers in the regulation of dibasic amino acid transport.

2. METHODS

2.1. Chemicals

Tritiated lysine (L-[4,5-^{3}H] lysine monohydrochloride) was purchased from Amersham Canada Ltd (Oakville, Ont., Canada). All amino acids, dibutyryladenosine 3':5'-cyclic monophosphate, 3-isobutyl-1-methyl-xanthine (IBMX), and bovine serum albumin were supplied by Sigma Ltd (St Louis, MO, U.S.A.). The 1,9-dideoxy forskolin and forskoline was supplied by Calbiochem Corp. (San Diego, CA, U.S.A.). N-(2-guanidinoethyl)-5-isoquinolinesulfonamide hydrochloride (HA1004) and 1-(5-Isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7) were purchased from Seikagaku America Inc. (St. Petersburg, FL, U.S.A.). Supplies for the cyclic nucleotide assay were a generous gift from Dr. A.K. Ho, Department of Physiology, University of Alberta, Alberta, Canada. The ^{125}I-cAMP and ^{125}I-cGMP label was purchased from Bionetic (Rockville, MD, U.S.A) and the antibodies to cAMP and cGMP were kindly provided by Dr. Albert Baukal (NICHD, NIH, Bethesda, MD, U.S.A.).

2.2. Stock Solutions

Forskolin and 1,9-dideoxy forskolin were solubilized in dimethyl sulfoxide (DMSO) to a concentration of 10 mM. HA-1004, and H-7 were solubilized in double-distilled water to

a concentration of 10 mM. IBMX was prepared in frog Ringer's solution to a stock concentration of 2.25 mM.

2.3. Vascularly Perfused Frog Preparation

2.3.1. Animals:

The animal used was the leopard frog, *Rana pipiens*- weight range 30-50 g, fed once weekly with live crickets and/or mealworms.

2.3.2. Operative procedure:

After pithing, the animal was placed upon a metal tray and the abdominal skin cut to expose the abdominal wall. Two incisions were made lateral to the abdominal vein to facilitate ligation. Once completed, the vein was removed. Both clavicles were cut through and the sternum removed. The vessels leading to and from the stomach and colon were tied off. At this point the major portion of the stomach was removed.

Displacement of the intestine and the liver exposed the systemic arches of the aorta and the coeliaco-mesenteric artery. After making the appropriate mesenteric windows loose ligatures were placed around the left systemic arch and the coeliaco-mesenteric artery. Exposure of the portal

vein allowed a loose ligature to be tied around the pancreas and vein where they are free from the liver.

Before inserting a cannula into the coeliaco-mesenteric artery via the left systemic arch, an incision was made in the portal vein at its junction with the vessels leading from the liver. A small incision was then made in the left systemic arch to facilitate insertion of the arterial cannula beyond the gastric branch of the coeliaco-mesenteric artery where it was firmly tied in place (See Figure 3). Immediately, a cannula was inserted into the portal vein and tied into place.

A cannula was inserted into the proximal end of the small intestine through the pylorus and likewise into the distal end of the small intestine at the junction with the colon. The intestine was then irrigated with a minimum of 30 mL of frog Ringer to ensure there were no obstructions to flow.

At this stage, the intestine had a closed vascular circulation separate from the rest of the animal and was cannulated permitting luminal flow. This preparation allowed experimental control of the composition of solutions bathing both the luminal and serosal poles of the enterocyte.

Single pass perfusion of the lumen of the intestine and the vascular bed was carried out using flow rates of 30 mL/h and 16 mL/h respectively. Frog Ringer's solution was of the following composition (mM): NaCl 93, KCl 5, $MgSO_4$ 1, $MgCl_2$ 0.8, NaH_2PO_4 2.15, Na_2HPO_4 0.85, $NaHCO_3$ 25, $CaCl_2$ 0.5.

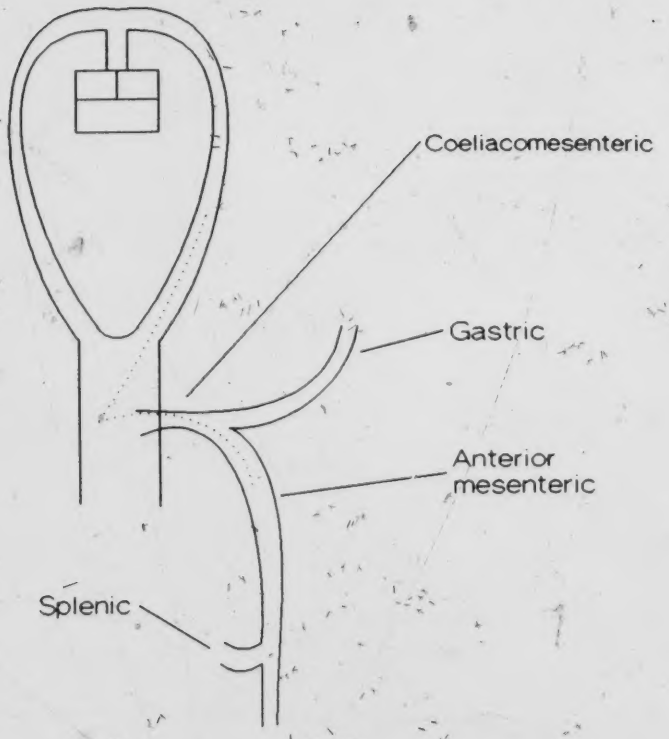

Figure 3. Anuran arterial vasculature. The arterial cannula (represented by the dotted line) is inserted in a small incision in the left aortic arch which arises from the heart. It is then advanced into the coeliaco mesenteric artery past the gastric branch and into the anterior mesenteric artery where it is tied into place.

Vascular perfusate had the identical composition but in addition contained 1 g/100 mL bovine serum albumin fraction V and 2 mM D-glucose.

Perfusion of the vascular bed and intestinal lumen was achieved via a multichannel Gilson Minipuls II peristaltic pump using nylon tubing of 0.76 mm I.D. and 1.42 mm I.D., respectively. Prior to use, the vascular perfusate was filtered through Whatman No. 1 filter paper to remove any particulate matter. Both the intestinal and vascular perfusate were continually gassed with 95% O_2/5% CO_2. The vascular perfusate was passed to a bubble trap located just before the arterial cannula. Collection of fluid from the portal vein was by means of a nylon tube. The end of this cannula was inserted into the portal vein and was not tapered but square-cut to prevent occlusion of the vein. This cannula was fitted into a tube leading to a Gilson Model 201 fraction collector. The vascular collection outlet was located below the level of the portal vein cannula in order to overcome the frictional resistance to flow in the narrow tube. This system is diagramatically represented in Figure 4.

2.3.3. Experimental Design:

The transmural transport of L-lysine is assayed by determining the rate of appearance of [^{3}H]-lysine in the vascular effluent collected via the cannula in the portal

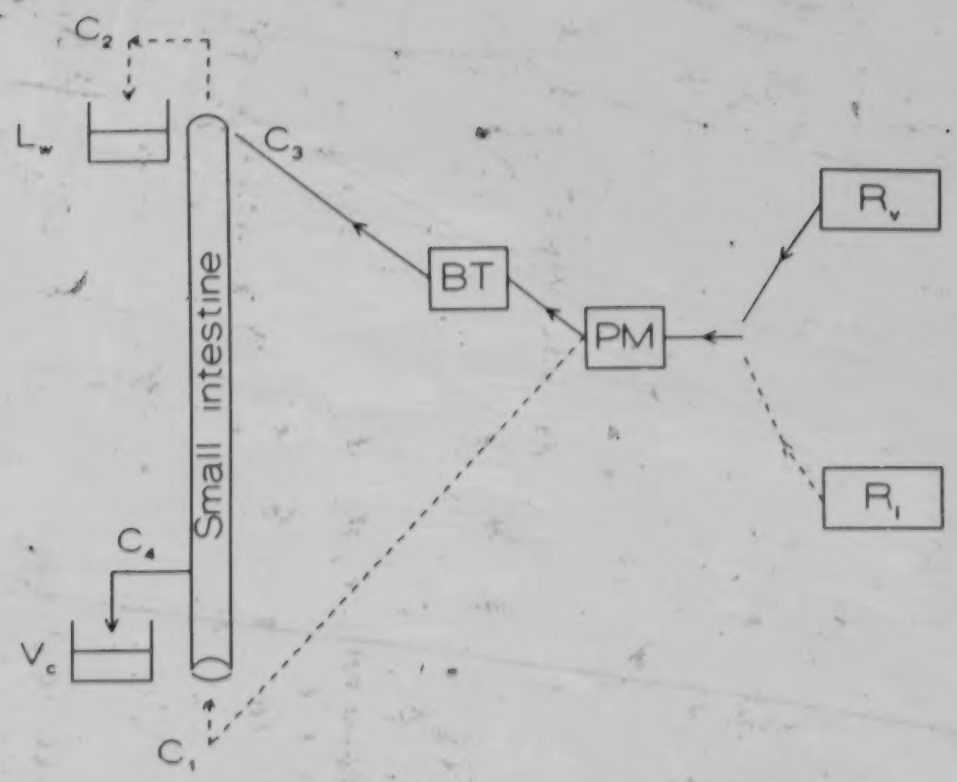

Figure 4. Diagramatic representation of frog vascular perfusion technique. R_V and R_1, vascular and luminal reservoirs. **PM** is both the vascular and luminal pump. **BT** is a bubble trap. C_1 and C_2, luminal cannulae; C_3, cannula in mesenteric artery; C_4, portal venous cannula; V_C, vascular collection with fraction collector; L_W, luminal waste collection. Modified from Boyd *et al.* (1975)

vein. The lumen of the intestine was continually perfused with 1 mM [^{3}]H-lysine/Ringer's.

2.3.4. Expression of Results:

The transmural rate of transport is expressed as μmoles of lysine appearing in the vascular effluent per gram dry weight of intestine per hour (μmol/g dry wt/h). To determine the dry weight of the intestine the tissue is excised and opened longitudinally along the antimesenteric surface. It is then weighed before and after drying to constant weight in a hot air oven.

2.3.4. Scintillation counting:

Aliquots of vascular effluent were added to a commercial scintillation cocktail (Scintiverse E, Fisher Scientific, Ottawa, Ont.) and counted in an LKB 1215 Rackbeta scintillation counter.

2.3.6. HPLC analysis of vascular perfusate:

The vascular perfusate collected after perfusion was collected and subjected to high performance liquid chromatographic analysis (HPLC). This work was conducted with the assistance of Mr. Michael Nattriss in the Dept. of Biochemistry, at the University of Alberta.

Briefly, 10 μL of vascular perfusate collected before and after stimulation with 1 mM leucine perfused through the vascular bed was collected. These samples were analyzed with an automated phenyl isothiocycanate (PITC) derivatization system from Applied Biosystems (Foster City, CA, U.S.A). The unit consisted of the 920 A Data Analysis Module, 130 A Separation System, and the 420 A Derivatizer. Samples were loaded manually onto the reverse phase column and detected with a U.V detector at 254 nm.

2.4. Cyclic Nucleotide Determinations

The effect of leucine on tissue cyclic nucleotide levels was determined employing a cyclic nucleotide radioimmunoassay procedure developed by Harper and Brooker (1975). To this point in time, cyclic nucleotide assays have been carried out on a variety of experimental samples. These include rings of frog intestine, isolated rat enterocytes, and mucosal scrapings of frog intestine, described below.

2.4.1. Rings:

Rings of frog intestine were prepared by quickly removing the intestine from a pithed frog , and using a scalpel blade to section rings approximately 2-3 mm in width. Prior to incubation in test solutions, rings were incubated in three

ten-minute changes of gassed frog Ringer's which had been supplemented with 2 mM of D-glucose . Tissue was then incubated in a 2 mL. volume of frog Ringer's with 2 mM of D-glucose and various combinations of amino acids or 3-isobutyl-1-methylxanthine (IBMX). After incubation, tissue was frozen on dry ice and sonicated with a Braun sonicator in one volume of acetic acid. At the same time, an aliquot of incubation medium was removed and placed on dry ice. The sonicated tissue was placed back on dry ice and then both the incubation medium and tissue were boiled for 5 minutes. The homogenized tissue was then centrifuged in a Beckman microfuge (800 x g) for 5 min and the supernatant subjected to the assay procedure.

Results are expressed as moles of cAMP or cGMP per ug of DNA. DNA was assayed as described by Holt et al. (1983). Briefly, 100 µL of the sonicated tissue was extracted in 0.5 mL of cold 0.8 M perchloric acid for 30 min, and centrifuged in a Beckman microfuge (800 x g) for two minutes. The pellet was washed with 0.5 mL of 0.4 M perchloric acid and recentrifuged, and washed again with 0.5 mL of 0.4 M perchloric acid and hydrolyzed at 70°C for 30 minutes. After cooling, 0.5 mL of Burton's reagent was added and colour was allowed to develop overnight. Burton's reagent consisted of 100 mL glacial acetic acid and 1.5 mL H_5SO_4 (Fisher) with 1.5 g of diphenylamine and 0.5 mL of glutaraldehyde. The absorbance of samples was determined at 600 nm in a Phillips spectrophotometer and compared against

a standard curve using calf thymus DNA as a standard. Reagents were from Sigma unless otherwise noted.

2.4.2. Isolated Cells:

2.4.2.1. Animals:

Male Sprague-Dawley rats (weight range 300-350 g) were used and given free access to food (rat chow) and water.

2.4.2.2. Preparation:

Isolated rat enterocytes were prepared according to the method of Cheeseman et al. (1985). Briefly, male Sprague-Dawley (200-250 g) rats were anesthetized with sodium pentobarbital, and the entire small intestine removed, everted, washed and incubated for 20 min in a gassed (95% O_2/5% CO_2solution of calcium-free Krebs bicarbonate saline containing 1.5 mg/mL hyaluronidase, 2.5 mg/mL albumin, and 2 mM sodium citrate and shaken at 80 strokes per minute in a water bath which maintained the temperature at 37°C. Every 5 minutes during the incubation the solution was agitated vigorously with a plastic pipette tip, a procedure which greatly increased the cell yield. The resulting suspension was then filtered through a nylon mesh to remove mucus and tissue debris before being centrifuged for 2 min at 400 x g

in a DYNAC II centrifuge (Clay Adams Co.). The cell pellet was resuspended in 30 mL of Krebs solution at 4°C, mixed thoroughly and recentrifuged for 2 minutes at 1200 rpm. This wash procedure was repeated to give a final yield ranging from 0.5 to 1.0 mL packed cells. Trypan blue exclusion by these cells was routinely better than 90% and often exceeded 95%. At all stages of the preparation, plastic beakers and test tubes were employed to minimize damage to the cells and to reduce aggregation.

Cells were used immediately after preparation. Incubation of cells was carried out in 1 mL total volume of incubation fluid (frog Ringer's and test compounds) at 37°C in a 95% O_2/5% CO_2 atmosphere. Incubation times and material subjected to the cyclic nucleotide assay varied.

Results are expressed as nmole cAMP per 100,000 cells. Cell density was estimated using a hemocytometer.

2.4.3. Mucosal Scrapings:

Frog intestines were perfused with 1 mM lysine through the intestinal lumen. Control preparations were perfused though the vascular bed with untreated vascular perfusate for a period of 30 minutes. Experimental conditions were invoked by perfusing the vascular bed with either 1 mM leucine or 1 μM forskolin for 30 minutes. After perfusion was completed, the intestine was quickly excised, opened longitudinally, and the mucosa removed with a glass slide.

The resultant scrapings were then quickly placed in microcentrifuge (1.5 mL) tubes pre-cooled on dry ice. An aliquot of 100 μL of acetic acid was then added to each tube and the resulting suspension sonicated with two 5 second pulses from a Braun Sonic 2000 sonicator set at an intermediate setting. The samples were then placed back on dry ice for 10 to 20 minutes and then placed in a boiling water bath for 5-7 minutes. The tubes were then centrifuged at 800 x g in a Beckman microfuge and the supernatent subjected to the assay procedure. Results are expressed as pmole cAMP or cGMP per mg protein. Protein levels were estimated by the method of Bradford (1976) using the Bio.Rad protein assay kit (Bio.Rad Laboratories, Richmond, CA, U.S.A.).

2.4.4. Cyclic nucleotide radioimmunoassay:

Cyclic AMP and cGMP were determined in duplicate by RIA, following an acetylation procedure (Harper and Brooker, 1975). [^{125}I]Succinyl-cAMP and [^{125}I]succinyl-cGMP and corresponding antisera (1:25,000) were incubated with standards and samples for 16-18 h. Sheep antirabbit serum (100 μL diluted 1:1 with buffer) and polyethylene glycol (M.W. 8000) (1 mL, 7.5 % wt/vol) were added to each tube, and the tubes were centrifuged (2,500 X g, 30 min, 2°C). Radioactivity in the pellet was determined. The range of each assay was 2.0-500 fmole cAMP or cGMP/tube. The cross-

reactivity of the cAMP antiserum for cGMP and the cGMP antiserum for cAMP was 0.07% and 0.41%, respectively. Intra- and interassay coefficients of variation were < 10%. All data are presented as the mean ± SEM. Statistical comparisons were done by the Student's t-test.

3. RESULTS

3.1. The Leucine Effect

Experiments on the vascularly perfused frog were designed to facilitate examination of the role of second messengers in the stimulation of transmural lysine transport by leucine. Prior to the initiation of such experiments, however, it was important to characterize the system and ensure that the leucine stimulation of lysine transport observed in previous work (Cheeseman, 1983) could be reproduced.

The stimulation of transmural lysine transport by leucine was demonstrated unfailingly. A typical experiment is represented in Figure 5. During the first 30 minutes, the basal steady state rate of lysine transport was established and in this particular experiment averaged approximately 10 μmol/g dry wt/hr. Stimulation of transport was achieved by perfusing frog Ringers with 0.5 mM leucine through the lumen of the intestine. As is apparent, stimulation of lysine transport occurs within the first 1-3 minutes of leucine perfusion and maximal stimulation is achieved within 5 minutes, after which a new steady state rate of transmural lysine transport is achieved at a slightly lower level which is maintained for the duration of leucine perfusion. When the lumenal perfusate is switched to one without leucine,

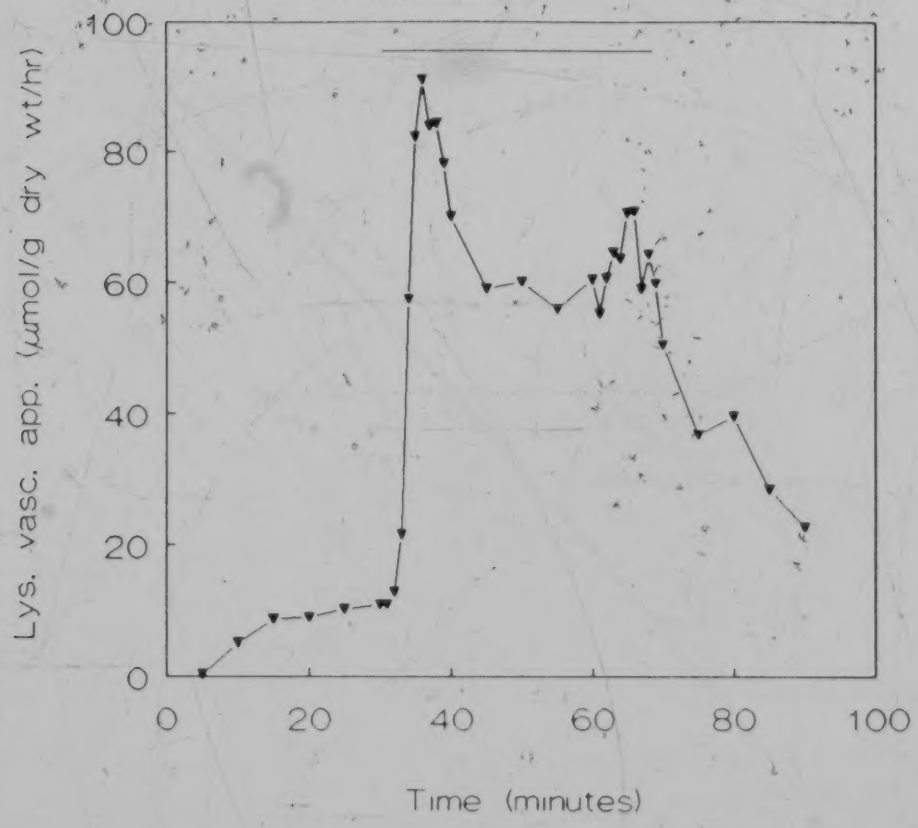

Figure 5. Effect of leucine on steady state transmural lysine transport. Leucine was perfused through the intestinal lumen at a concentration of 0.5 mM for the period indicated by the bar. Lys. vasc. app. = Lysine vascular appearance in portal effluent.

the rate of lysine transport returns to normal within about 30 minutes.

The response to leucine is stereospecific (Figure 6). When 0.5 mM D-leucine is perfused through the intestinal lumen it stimulates lysine transport only slightly when compared to the stimulation incurred by the same concentration of L-leucine. Note that the stimulation by D-leucine is very gradual in onset, not abrupt like the stimulation by L-leucine.

Further characterization of this phenomenon demonstrated that it is dose-dependent (Figure 7). In this instance leucine at varying concentrations (0.01, 0.1 and 1.0 mM) was perfused through the vascular bed of the intestine. As before, the response to leucine is very rapid in onset, occurring within 1 minute of leucine perfusion. The dose-response curve of lysine transport to leucine will be presented in more detail subsequently.

To demonstrate that the previous findings are not the result of a time-dependent change in lysine transport, the basal rate of lysine transport was monitored over a ninety minute period (Figure 8). It is apparent that the steady state rate of transmural lysine transport remains relatively constant during the duration of the experiment. There is a tendency for a slow increase in the rate of transport to occur as the experiment progresses but this is not significant compared with the large degree of stimulation observed in the presence of leucine.

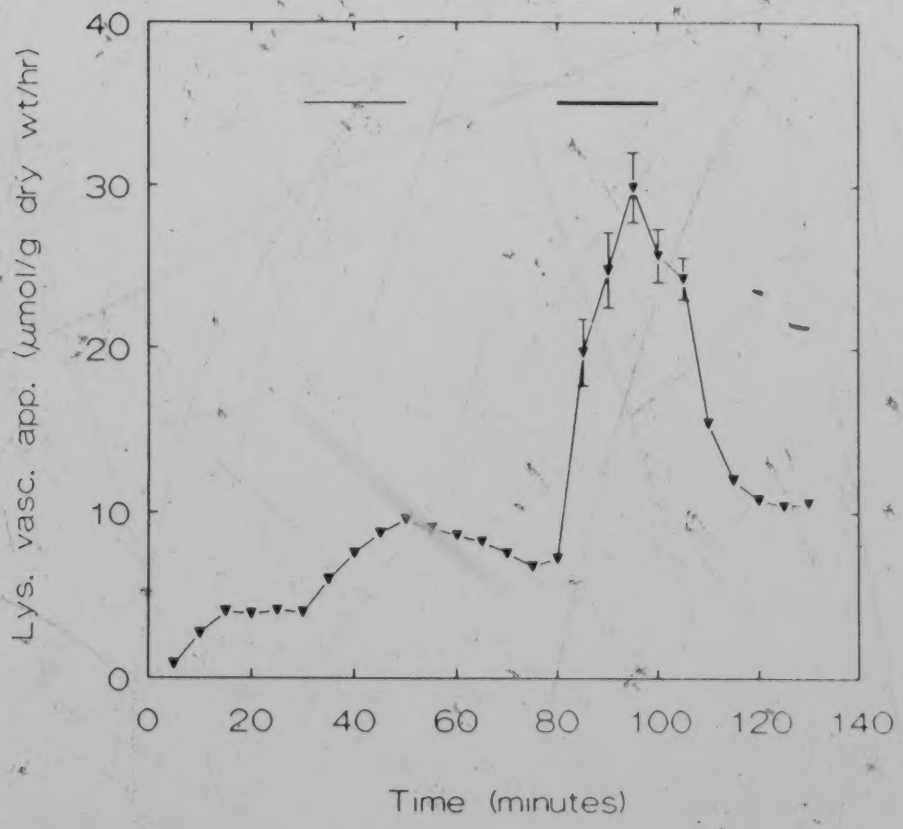

Figure 6. Stereospecificity of the response of steady state transmural lysine transport to stimulation by leucine. D- and L-leucine were perfused through the intestinal lumen at a concentration of 0.5 mM. D- and L-leucine were perfused for the period represented by the light and heavy bars, respectively. Each point is the mean of 3 determinations ± SEM.

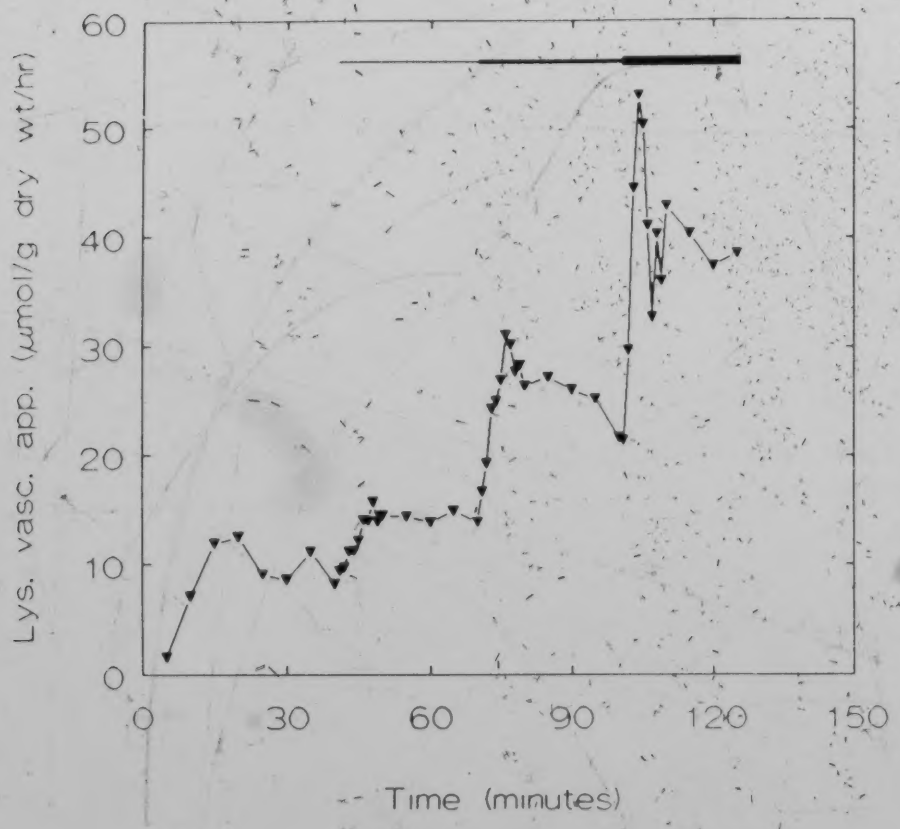

Figure 7. Dose-response of steady state transmural lysine transport to leucine stimulation. Leucine at 0.01, 0.1, and 1.0 mM was perfused through the vascular bed at the times represented by the light, medium and heavy bars, respectively.

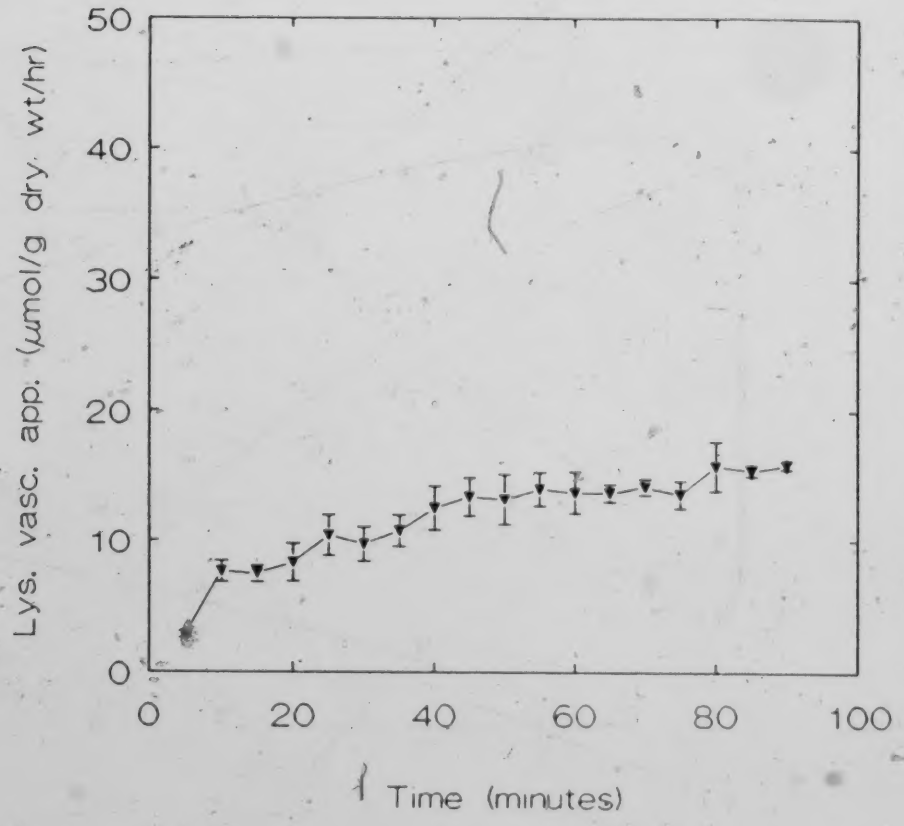

Figure 8. Steady state transmural lysine transport as a function of time. Each point represents the mean of three determinations ± SEM.

Cheeseman (1983) determined that the frog intestine metabolizes lysine at a rate of approximately 10% per hour. This raises the very real possibility that the increased rate of appearance of radioactive substrate we see in the vascular perfusate in response to leucine may not represent the transport of lysine across the basolateral membrane but that of a breakdown product of lysine. Perhaps leucine in some way switches on the catabolism of lysine, the products of which then can leave the cell via a mechanism with a higher, intrinsic rate of transport. To examine this possibility the change in lysine appearance in identical fractions of vascular perfusate were analyzed using radioisotope methodology, and high performance liquid chromatography. As is revealed in Figure 9 there is no statistical difference between the change in lysine transport invoked by leucine as determined by radioisotope procedures or by HPLC. Leucine invoked a 8.1 ± 1.08- fold change in lysine appearance as determined with radiolabelled lysine (i.e. the standard method of assessing this response) and a 10.2 ± 1.49- fold change as determined by HPLC. This, for the first time, clearly demonstrates that, indeed, leucine is not affecting lysine metabolism but is changing the rate at which lysine enters the vascular solution.

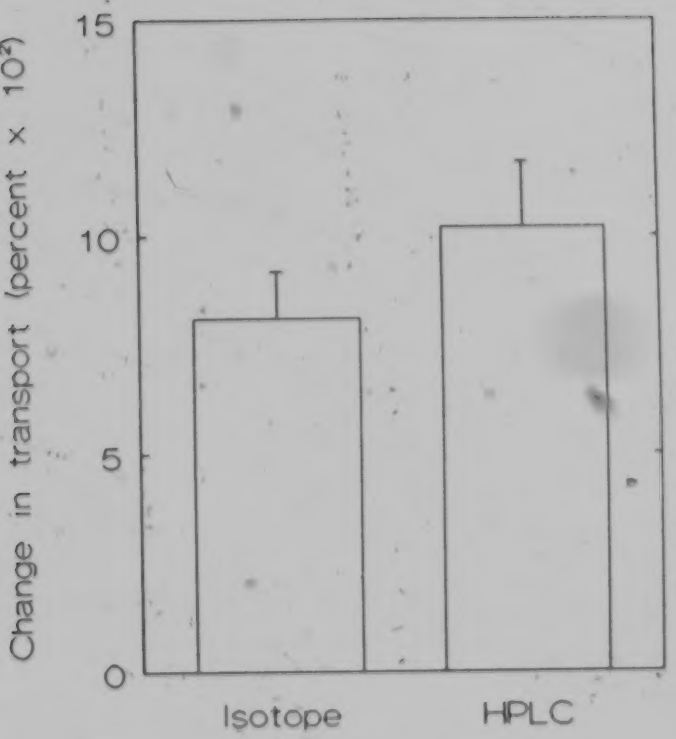

Figure 9. Change in steady state transmural lysine transport when stimulated by leucine (1 mM) as determined by radioisotope methodology or HPLC. Each bar represents the mean of 3 determinations ± SEM.

3.2. IBMX, forskolin, and DbcAMP: Effects on transmural lysine transport.

I chose to begin the examination of the effect of second messengers on lysine transport by examining the role of cyclic nucleotides (cAMP, cGMP). This was facilitated by the use of three compounds. These were 3-isobutyl-1-methyl xanthine (IBMX) a phosphodiesterase inhibitor (Weishaar, 1987), forskolin, a plant diterpene with the ability to directly stimulate the activity of adenylate cyclase (Seamon and Daly, 1986), the enzyme responsible for cAMP generation, and dibutyryl cAMP, a lipid-soluble analog of cAMP that retains cAMP activity.

3.2.1. Effect of IBMX on transmural lysine transport:

The effect of IBMX on the leucine stimulation of lysine transport is represented in Figure 10. Basal steady state lysine transport is established between 9-10 μmole/g dry wt/hr. Leucine (0.5 mM) perfused through the vascular bed stimulates transmural transport approximately 2-fold. When leucine is withdrawn from the system, transport rapidly declines to basal levels. Perfusion of the intestinal lumen with 50 μM IBMX while simultaneously perfusing the vascular bed with leucine (0.5 mM) stimulated lysine transport almost four-fold above baseline. This was the first indication that cyclic nucleotides might be involved in the response of

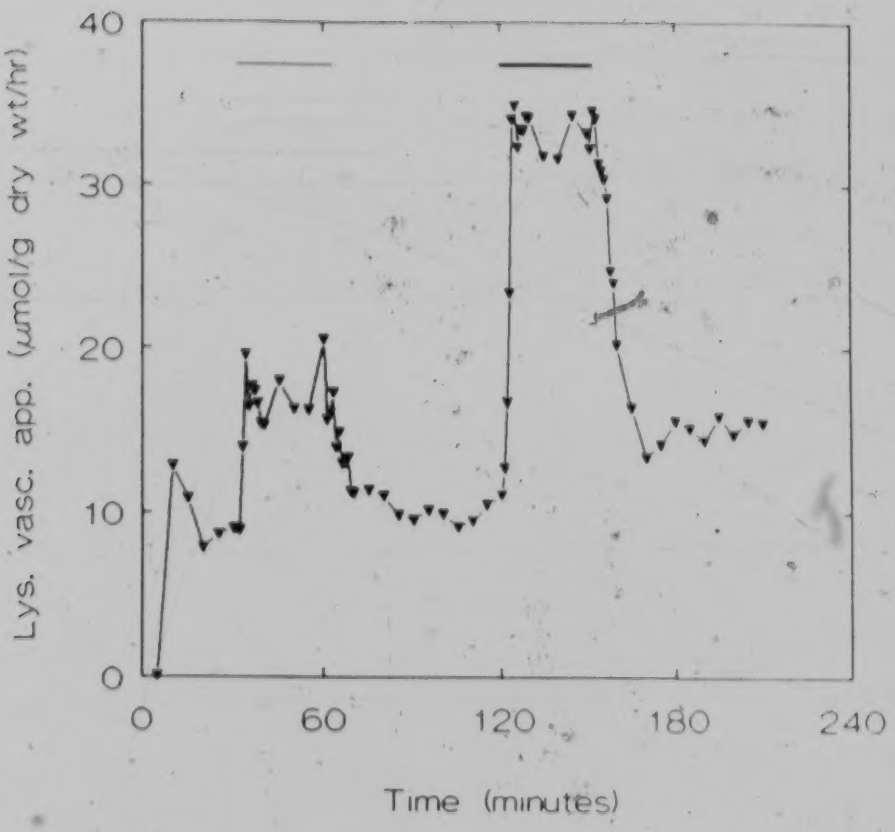

Figure 10. Leucine (0.5 mM) was perfused through the vascular bed for the period indicated by the light bar. Leucine (0.5 mM) was perfused with simultaneous perfusion of 50 μM IBMX through the intestinal lumen for the period indicated by the heavy bar.

lysine transport to leucine. The rationale is obvious: if leucine is stimulating lysine transport via a second messenger such as cAMP, then inhibiting the catabolism of cAMP by phosphodiesterase with IBMX should produce an enhanced response to leucine. This is what was observed.

Several control experiments were conducted of which Figure 11 depicts a representative experiment from this series. Four experiments identical to the one represented in Figure 11 were completed. Basal transport was 8.45 ± 0.16 μmol/g dry wt/hr. The initial pulse of leucine (1 mM) elevated lysine transport to 28.45 ± 0.78 μmol/g dry wt/hr and the second leucine pulse elevated transport to 29.13 ± 0.47 μmol/g dry wt/hr. Perfusion of IBMX (50 μM) alone through the intestinal lumen had no effect on the basal rate of transport. Simultaneous perfusion of IBMX (50 μM) and leucine (1 mM) through the intestinal lumen elevated lysine transport to 49.46 ± 0.47 μmol/g dry wt/hr. It is clear from these data that two successive pulses of leucine (1.0 mM) through the vascular bed produce the identical level of stimulation of lysine transport ($p < 0.05$). This is important because it means that the IBMX potentiation noted previously (Figure 10) cannot be the result of an initial leucine pulse potentiating the response to a second such pulse. Further, IBMX perfusion through the intestinal lumen in the absence of leucine perfusion has no effect. It is only when leucine and IBMX are perfused simultaneously that the potentiation of transport occurs ($p < 0.05$).

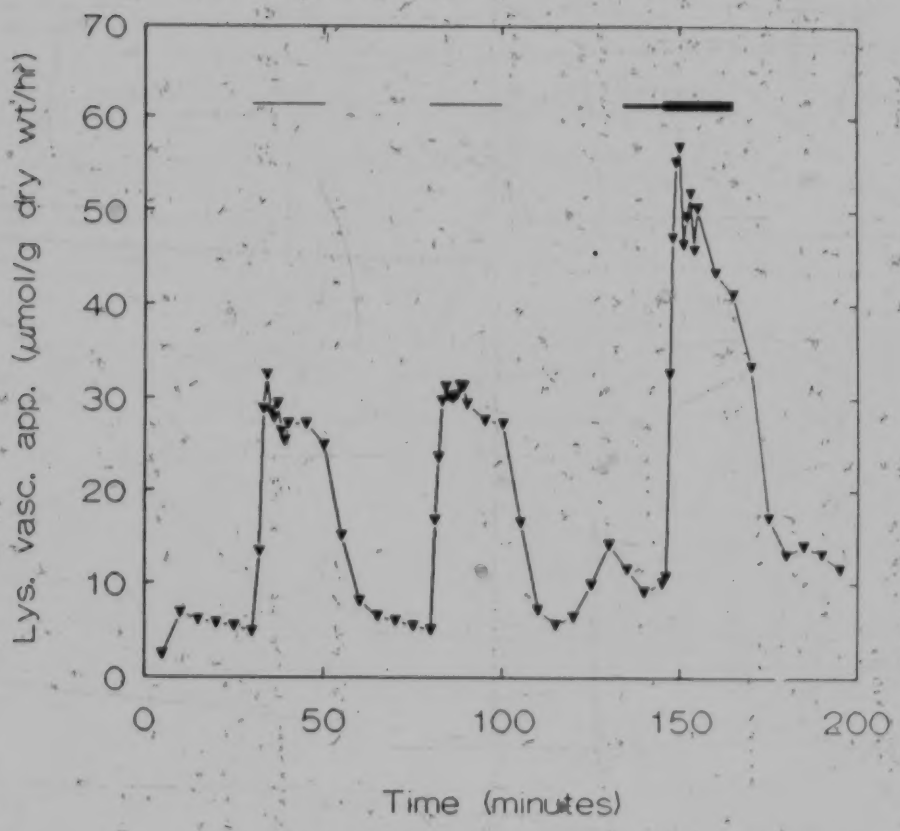

Figure 11. Effect of dual pulses of leucine (1 mM) and leucine with 50 μM IBMX perfusion through the intestinal lumen on the steady state appearance of lysine in the vascular bed. Leucine alone (light bar), IBMX alone (medium bar) and leucine plus IBMX (heavy bar). Each point represents the mean of three determinations ± SEM.

A dose response curve of lysine transport to varying concentrations of leucine was constructed in both the absence and presence of IBMX (Figure 12). Lysine transport was stimulated by 0.01, 0.1, and 1.0 mM leucine perfused through the vascular bed to the extent of 1.35-, 2.68-, and 3.76- fold, respectively. With simultaneous perfusion of 50 μM IBMX through the intestinal lumen lysine transport was stimulated by the same concentrations of leucine to 2.95-, 5.73-, and 10.14- fold, respectively. This shifted the entire dose-response curve to the left. Clearly, IBMX is capable of potentiating the stimulation of lysine transport at several different concentrations of leucine.

This evidence strongly suggested a possible role for cyclic nucleotides in the leucine stimulation of lysine transport. Therefore, to strengthen the hypothesis, it is important to demonstrate that increased cyclic nucleotide levels in the tissue is capable of stimulating transmural lysine transport. This could be achieved in a number of ways. I chose initially to try the most direct route and perfuse the intestinal lumen with frog Ringers to which had been added DbcAMP.

3.2.2. Effect of DbcAMP on transmural lysine transport:

DbcAMP (2.5 mM) appears capable of stimulating steady state transmural lysine transport (Figure 13). Note, however, that this effect is longer in onset and does not begin to

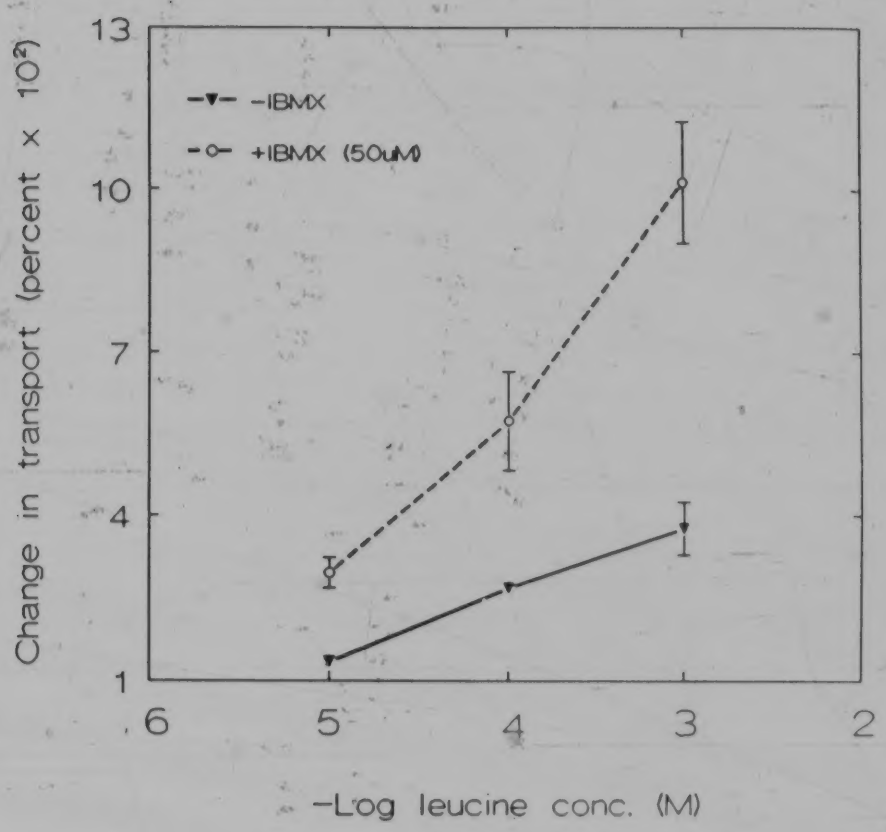

Figure 12. Effect of leucine and IBMX on lysine transport. Each point represents the mean of three determinations ± SEM of the increase in transport produced by 0.01, 0.1, and 1.0 mM leucine in the vascular bed with and without IBMX (50 μM) perfusion through the intestinal lumen.

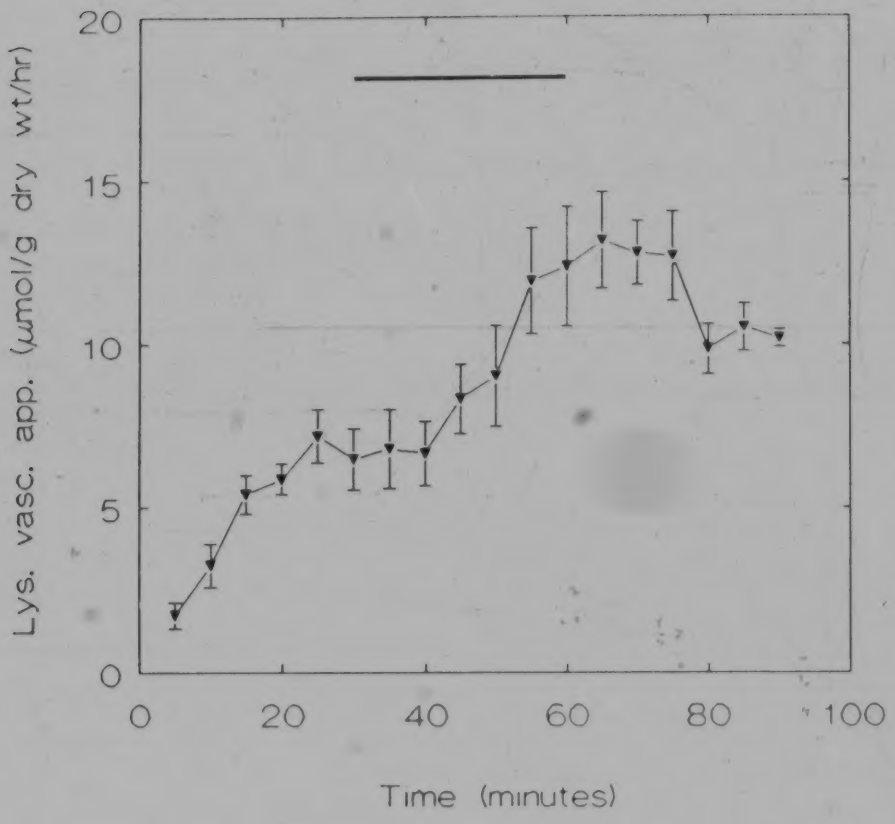

Figure 13. Effect of dibutyryl cAMP (2.5 mM), when perfused through the vascular bed, on steady state transmural lysine transport. DbcAMP was included in the vascular perfusate for the period indicated by the bar.

decrease immediately upon switching the vascular perfusate to one without DbcAMP. Indeed, it seems that the entire response is slightly out of phase compared to the stimulation of lysine transport obtained with leucine.

3.2.3. Effect of forskolin on transmural lysine transport:

As a third test for the possible involvement of cyclic nucleotides, the vascular bed of the frog was perfused with 1.0 μM forskolin. Forskolin is a plant diterpene which has the ability to directly stimulate adenylate cyclase , the enzyme responsible for cAMP synthesis (Seamon and Daly, 1986).

It is evident that forskolin when perfused through the vascular bed is capable of stimulating lysine transport (Figure 14). Transport achieved a new steady state (2.3-times the basal value) within 10-15 minutes of initiation of forskolin perfusion and began to return to basal levels when perfusion with forskolin was terminated. A dose-response curve of lysine vascular appearance to forskolin is shown in Figure 15. The response of lysine vascular appearance to increasing concentrations of forskolin is clearly dose-sensitive. Note, however, that the level of stimulation is nowhere near the level that can be obtained with leucine.

A number of competition experiments were carried out (i.e. arginine competing for lysine transport) in order to investigate the manner of forskolin stimulation of lysine

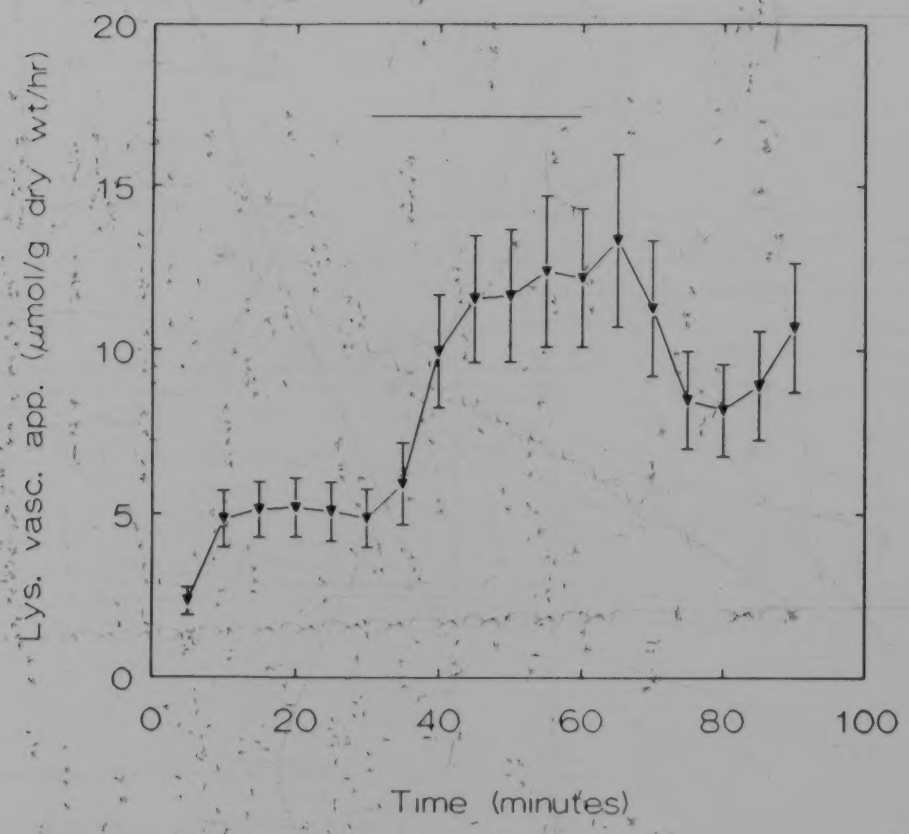

Figure 14. Response of steady state transmural lysine transport to 1.0 μM forskolin perfused through the vascular bed. Forskolin was included in the vascular perfusate for the period indicated by the bar. Each point is the mean of three determinations ± SEM.

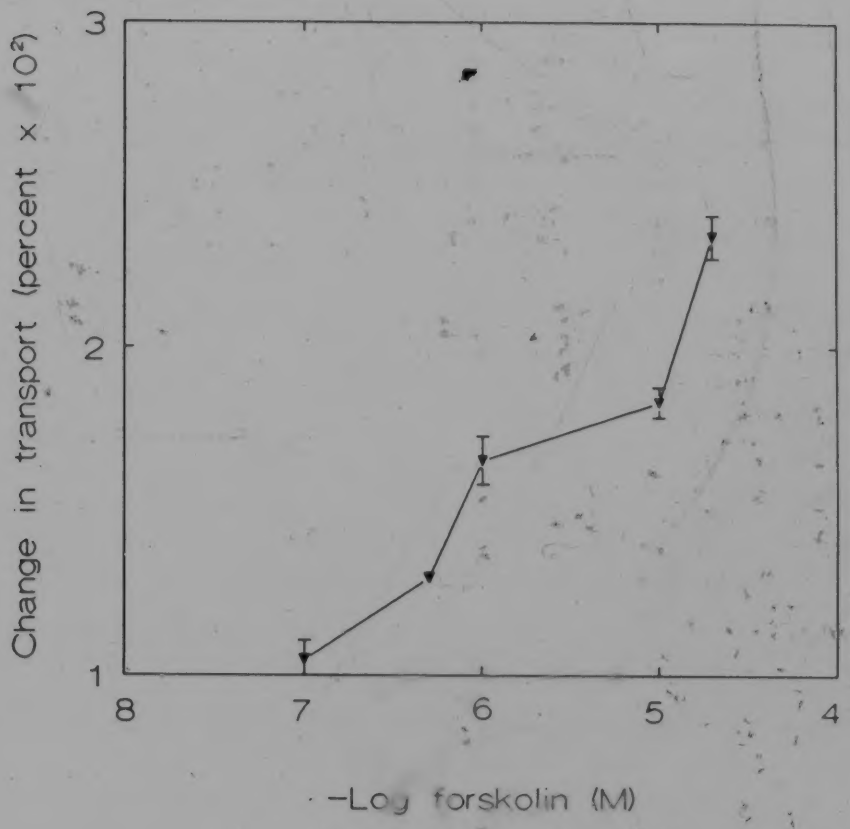

Figure 15. Relative increase in the rate of steady state transmural lysine transport as incurred by varying doses of forskolin. Each point represents the mean ± SEM of four detemination**s.**

transport (see Discussion for details). Figure 16 demonstrates that at least 80% of the transmural lysine transport being measured in the vascularly perfused frog preparation is attributable to specific transport processes (i.e. 50 mM arginine inhibited transport of 0.1 mM lysine approximately 80 %). When this preparation is then exposed to forskolin, it is evident that transmural lysine transport is not stimulated.

I have conducted experiments with the forskolin derivative 1,9-dideoxy forskolin (DDF) which is incapable of stimulating the production of cAMP in order to determine if the forskolin stimulation of lysine transport is a cAMP-dependent event. Experiments conducted (Figure 17a, 17b) indicate that DDF is not able to produce stimulation of transmural lysine transport. Each of the experiments with DDF was positively controlled by demonstrating that each preparation used was responsive to leucine perfusion through the vascular bed. Clearly, leucine markedly stimulated lysine transport in each preparation. This is important because it means that the mechanism of leucine stimulation is intact in each preparation and the failure to demonstrate DDF stimulation of lysine transport (assuming forskolin works through the same mechanism) is not due non-responsive preparations.

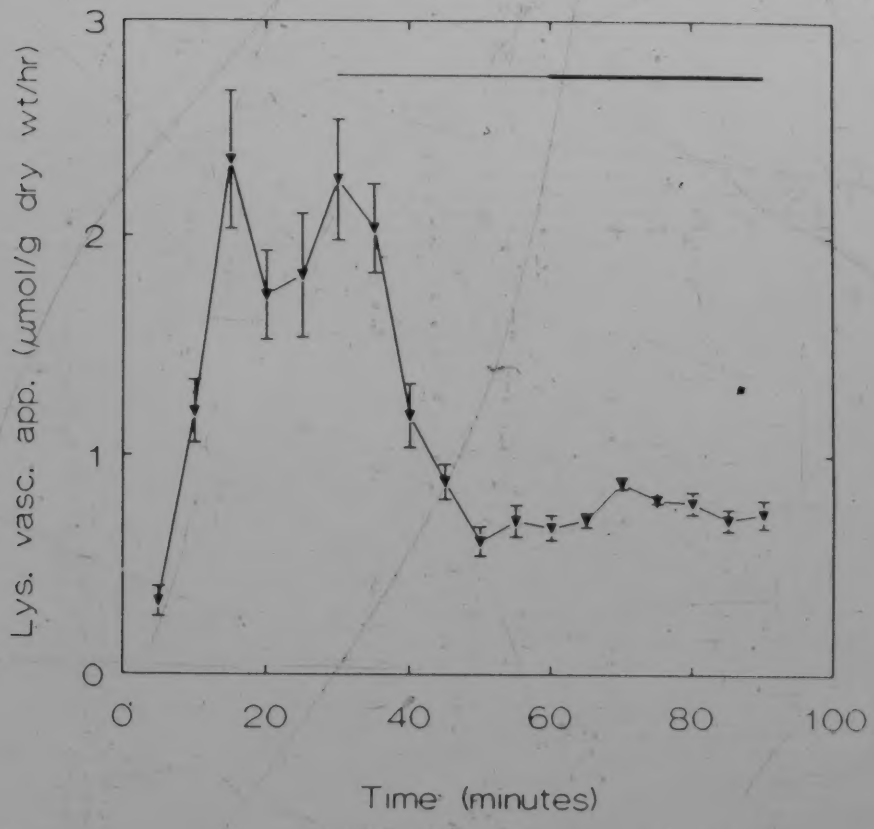

Figure 16. Effect of 1.0 μM forskolin on steady state transmural lysine transport when subjected to competition from 50 mM arginine. Arginine was perfused through the intestinal lumen for the period indicated by the light bar. Arginine was perfused through the intestinal lumen and forksolin was perfused through the vascular bed for the period indicated by the heavy bar. Each point represents the mean ± SEM of four determinations.

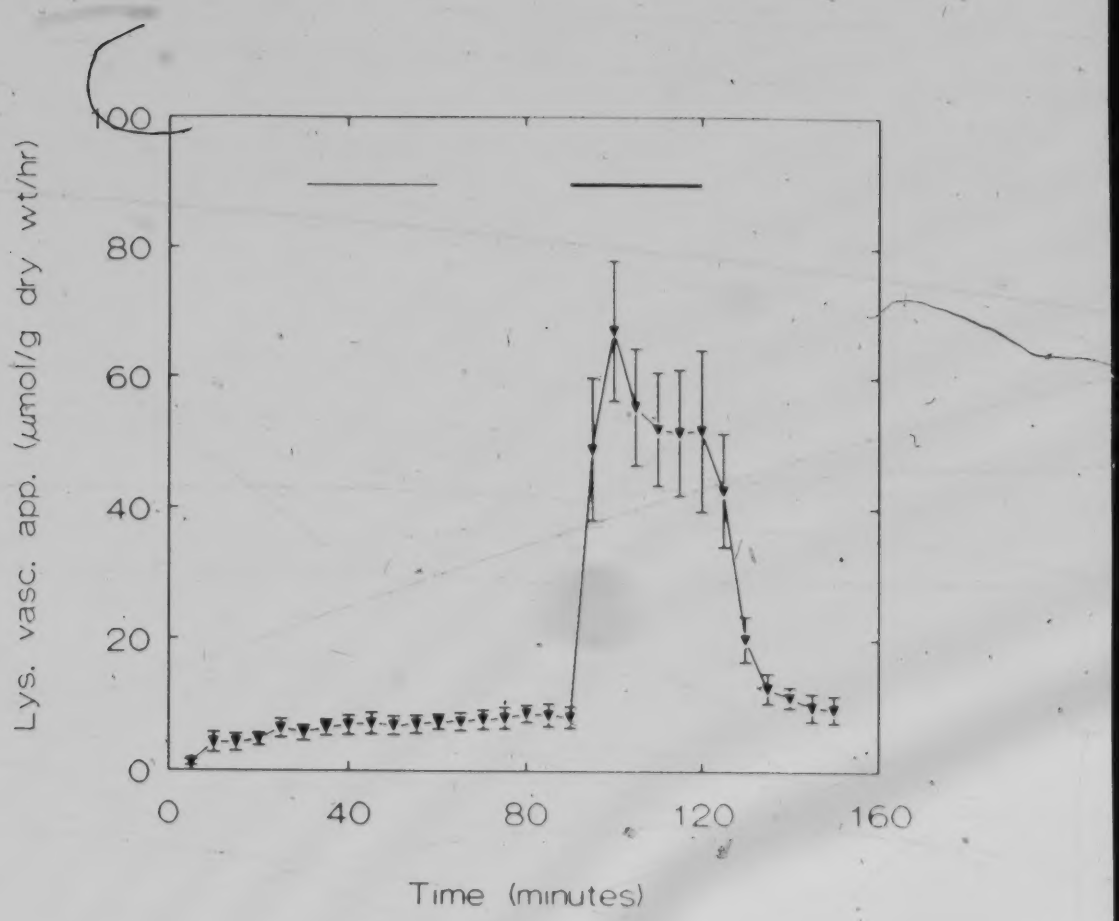

Figure 17a. Effect of 1,9-dideoxy forskolin on steady state transmural lysine transport. 1,9-dideoxy forskolin (1 μM) was perfused through the vascular bed for the period indicated by the light bar. Leucine (1 mM) was perfused through the vascular bed for the period indicated by the heavy bar. Each point represents the mean ± SEM of three determinations.

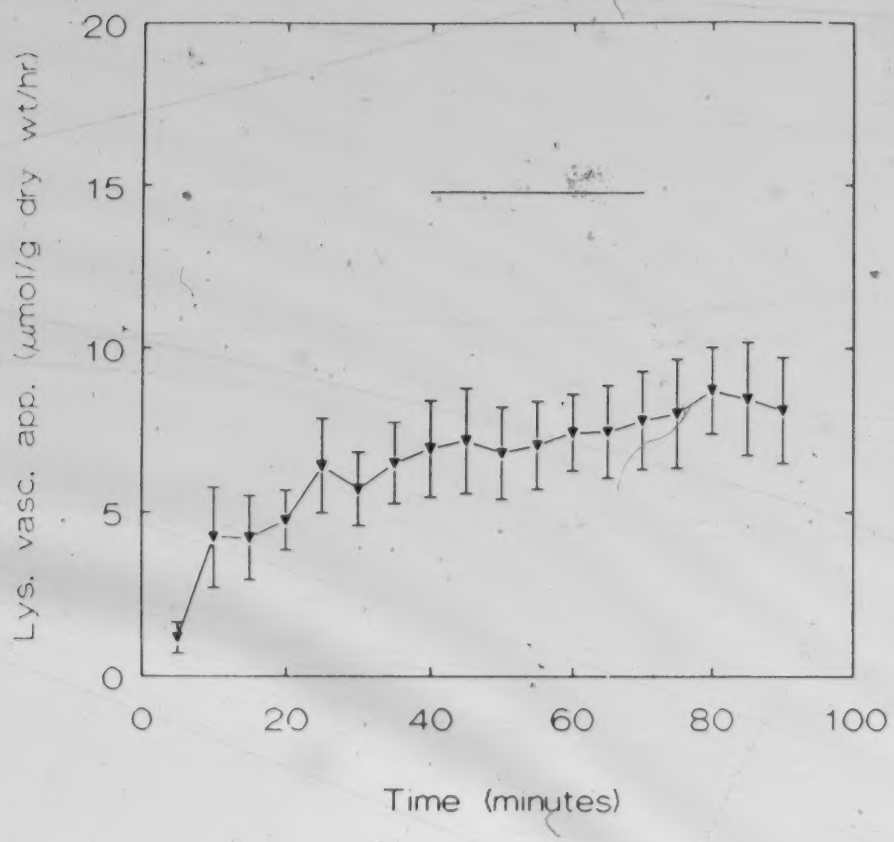

Figure 17b. Expansion of Figure 17a. 1,9-dideoxy forskolin was perfused for the period indicated by the bar. Each point represents the mean ± SEM of three determinations.

3.3. Cyclic nucleotide levels: Effect of leucine

Given the evidence from the aforementioned experiments, I felt safe in concluding that cyclic nucleotides play some role in the stimulation of transmural lysine transport by leucine. In order to conclude that a cyclic nucleotide (i.e. cAMP) is the second messenger responsible for the leucine effect, it must be demonstrated that leucine can directly influence the cAMP levels in the mucosal tissue. Rings of everted frog intestine, mucosal scrapings from frog intestine, and isolated enterocytes from rat jejunum were used in an attempt to determine if leucine was capable of elevating tissue cyclic nucleotide levels.

3.3.1. Effect of leucine on cyclic nucleotide levels in intestinal rings:

Table 2 represents the effect of 1 mM leucine, IBMX (50 μM), and leucine plus IBMX on the cAMP and cGMP levels in frog intestinal rings. Basal levels of cAMP were 331.76 ± 62.30 fmole/μg DNA. Exposure to leucine for 30 minutes did not significantly alter the concentration of cAMP in the rings ($p < 0.05$). Treatment with 50 μM IBMX increased cAMP levels to 653 ± 111.49 fmole/μg DNA. Simultaneous exposure of rings to IBMX and leucine yielded an average cAMP level of 925 ± 264.89 fmole/μg DNA, yet this was not significantly different from samples treated with IBMX only.

TABLE 2

Effect of leucine and IBMX on the cAMP and cGMP contents of rings of frog intestine.

Rings, 2-3 mm in width were quickly prepared from freshly excised frog intestine. Four rings per treatment were incubated for a test period of 30 minutes in oxygenated medium. All treatments were incubated in frog Ringers + 2 mM D-glucose plus one of the following: Control (no additions), Leu (1 mM leucine), IBMX (50 μM IBMX), Leu/IBMX (μM IBMX + 1mM leucine). Each value represents the mean ± SEM of cAMP or the cGMP content of three distinct experiments.

Treatment	Nucleotide Concentration *(fmole/μg DNA)*	
	cAMP	cGMP
Control	331.76 ± 62.30	31.68 ± 5.93
+ Leu	249.74 ± 37.18	27.89 ± 5.35
+ IBMX	653.37 ± 111.49[a]	41.04 ± 6.12[a]
+ Leu/IBMX	925.09 ± 264.89[a]	45.36 ± 16.03[a]

[a]Significantly different from the control group ($p < 0.05$).

Basal cGMP levels were 31.68 ± 6.35 fmole/μg DNA. Leucine had no significant effect on the cGMP accumulated by the rings. IBMX (50 μM) produced an increase in the average level of cGMP in the rings (41.04 ± 6.55 fmole/μg DNA) but this was not significant at 95 % level. When exposed to both leucine and IBMX the average level of cGMP remained elevated but again was not significantly different from that for IBMX alone.

3.3.2. Effect of leucine on isolated rat jejunal enterocytes:

Basal cAMP levels in isolated enterocytes were 1.2 ± 0.158 pmole/100,000 cells (Table 3). Leucine did not produce a significant change in cellular cAMP levels. Addition of 100 μM IBMX to the incubation medium produced a significant ($p < 0.05$) 2.5- fold increase in cAMP content of the cells. Leucine, at 5 mM with 100 μM IBMX, did not increase the cellular cAMP level above that observed with IBMX alone. Cyclic GMP was not detectable in this preparation.

3.3.3. Effect of leucine perfusion on cyclic nucleotide levels in mucosal scrapings:

Basal cyclic nucleotide levels in mucosal scrapings from anuran intestine that had been perfused for 30 minutes with

TABLE 3

Effect of leucine and IBMX on the cAMP content of isolated enterocytes.

Enterocytes were isolated from rat small intestine and incubated in Krebs bicarbonate. Cells were incubated for 30 minutes with 1 mM leucine, 5 mM leucine, 100 μM IBMX or 100 μM IBMX + 5 mM leucine at 37°C in a 95% O_2/ 5% CO_2 atmosphere. Each value represents the mean ± SEM of nine experiments.

Treatment	cAMP Concentration
	pmole/100,000 cells
Control	1.20 ± 0.16
+ Leu (1 mM)	1.39 ± 0.23
+ Leu (5 mM)	1.25 ± 0.14
+ IBMX	2.82 ± 0.58[a]
+ IBMX +Leu	3.05 ± 0.67[a]

[a]Significantly different from the control group ($p < 0.05$).

lysine through the intestinal lumen averaged 4.40 ± 0.46 pmole/mg protein (Table 4). Perfusion via the vascular bed with 1 mM leucine did not alter this value significantly. Forskolin perfusion (1 μM) although apparently causing some elevation in cAMP levels did not result in a significant difference from basal values. The effect of the same treatments on cGMP quantities was similar to those for cAMP. However, it is interesting to note that the drastic difference in the cAMP/cGMP ratio observed in the previous two preparations is not observed in this preparation.

3.4. Effect of HA-1004 and H-7 on transmural lysine transport.

HA-1004 and H-7 are compounds which are capable of inhibiting protein kinase activity. These compounds were utilized to investigate the possible role of protein kinases in the stimulation of lysine transport by leucine in the anuran intestine. Experiments conducted using HA-1004 are summarized in Figure 18. Initial stimulation by 1 mM leucine is apparent and elevates the basal transport rate from 3.5 ± 0.406 to 19.19 ± 1.122 μmol/g dry wt/h. Simultaneous perfusion of HA-1004 (100 μM) and 1 mM leucine through the vascular bed significantly ($p < 0.05$) increased the steady state rate of transmural lysine transport to 36.52 ± 2.903 μmol/g dry wt/h. Note that perfusion of HA-1004 by itself had no effect on transport. These findings

TABLE 4

Effect of leucine and forskolin on the cAMP and cGMP contents of mucosal scrapings of frog intestine.

Small intestines of frogs were perfused with 1 mM lysine through the intestinal lumen. Each preparation was perfused for a total of 60 minutes, the first 30 minutes of which were used to establish basal, steady state conditions. Control preparations were perfused through the vascular bed with untreated vascular perfusate for the latter 30 minutes. The "+ Leu" represents preparations which were perfused through the vascular bed with 1 mM leucine for the latter 30 minutes and "+ Forsk" were perfused with 1 μM forskolin. Each value represents the mean ± of four experiments.

Treatment	Nucleotide Concentration *(pmole/mg protein)*	
	cAMP	cGMP
Control	4.40 ± 0.46	6.68 ± 0.22
+ Leu	4.43 ± 0.58	7.36 ± 0.34
+ Forsk	5.64 ± 1.15	9.07 ± 1.86

Values between treatments are not significantly different ($p < 0.05$).

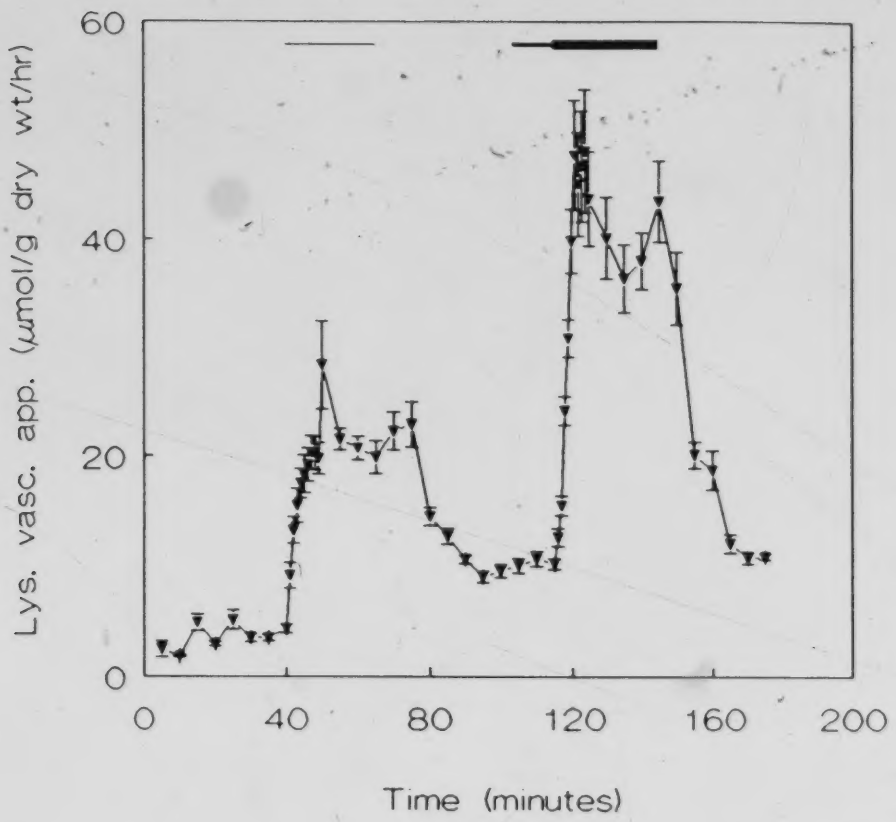

Figure 18. Leucine and HA-1004 perfusion through the vascular bed. Leucine (1 mM) was perfused through the vascular bed for the period indicated by the light bar. HA-1004 (100 μM) was perfused through the vascular bed for the period indicated by the medium bar. Leucine and HA-1004 were perfused simultaneously through the vascular bed for the period indicated by the heavy bar. Each point represents the mean of 4 determinations ± SEM

are contrary to what would be expected if leucine was stimulating lysine transport via cAMP.

Experiments with H-7 are preliminary. A typical experiment is represented in Figure 19. In this one experiment 1 mM leucine stimulated transport approximately 5- fold (i.e. from 2.61 ± 0.12 to 13.03 ± 0.969 μmol/g dry wt/h). Similar to the results with HA-1004, when leucine is perfused simultaneously with H-7 (100 μM), the transport of lysine is elevated significantly ($p < 0.05$) above that caused by leucine alone to 37.01 ± 2.90 μmol/g dry wt/h). Perfusion of H-7 had no noticeable effect on transport when perfused in the absence of leucine.

3.5. Effect ~~of~~ a substimulatory dose of forskolin on leucine stimulation of lysine transport.

In this series of experiments the effect of a substimulatory dose of forskolin (0.01 μM - see Figure 15) on the leucine stimulation of lysine transport was investigated. The experiments are similar in format to the previous experiments with the isoquinolinesulfonamide derivatives, HA-1004 and H-7.

Since forskolin was solubilzed in DMSO it was important to investigate whether DMSO on its own had any effect on lysine transport. Leucine elevated lysine transport to 16.99 ± 1.27 μmol/g dry wt/h in the absence of DMSO. When stimulation by leucine occurred with the appropriate volume

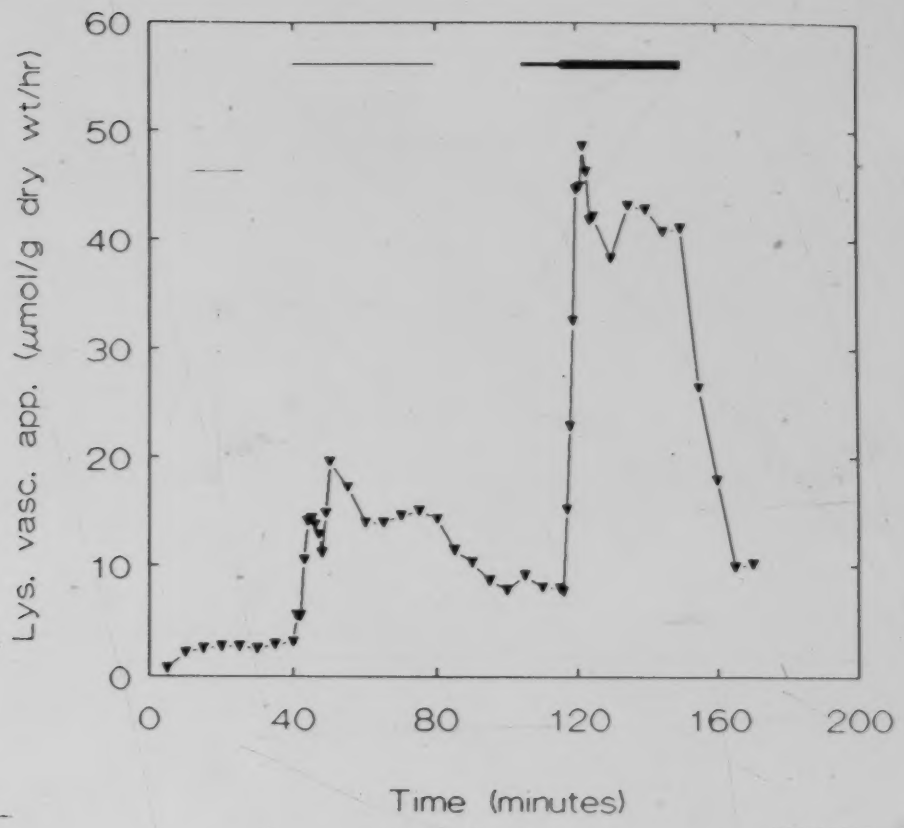

Figure 19. Leucine and H-7 perfusion through the vascular bed. Leucine (1 mM) was perfused through the vascular bed for the period indicated by the light bar. H-7 (100 μM) was perfused through the vascular bed for the period indicated by the medium bar. Leucine and H-7 were perfused simultaneously through the vascular bed for the period indicated by the heavy bar.

of DMSO added to the vascular bed the transport rate elevated to 16.40 μmol/g dry wt/h, a level not significantly different from the previous value ($p < 0.05$) (Figure 20).

Contrary to these findings, when 0.01 μM forskolin was included in the vascular perfusate, the stimulation caused by leucine increased. Basal transport was elevated by 1 mM leucine from 2.25 ± 0.431 to 12.02 ± 0.754 μmol/g dry wt/h. With forskolin in the vascular perfusate the same concentration of leucine elevated transport to 17.97 ± 1.29 μmol/g dry wt/h (Figure 21).

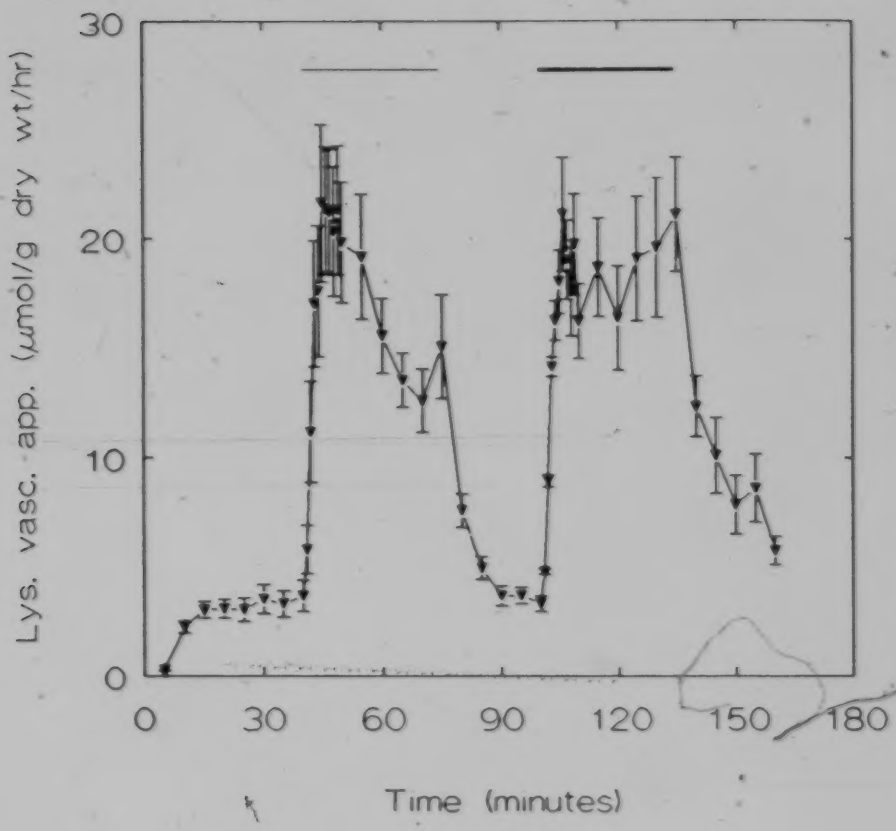

Figure 20. Effect of dual pulses of leucine (1 mM) on the steady state appearance of lysine in the vascular bed. Leucine alone (light bar) and leucine with DMSO (heavy bar) were perfused through the vascular bed for a period of 30 minutes. Each point represents the mean ± SEM of three determinations.

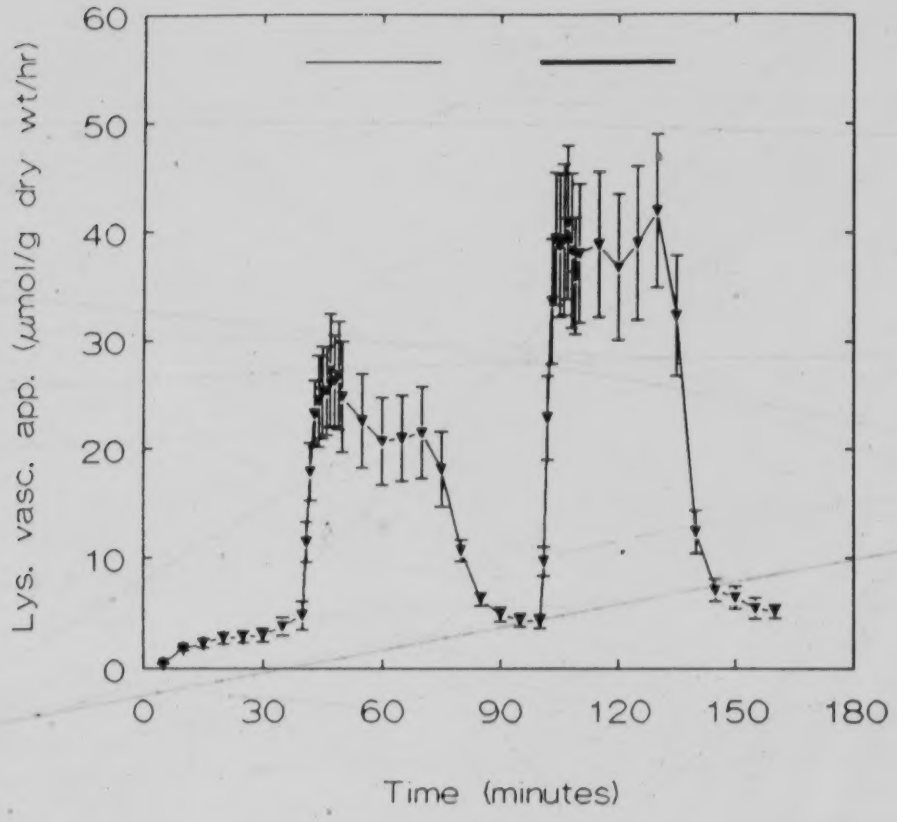

Figure 21. Effect of dual pulses of leucine (1 mM) on the steady state appearance of lysine in the vascular bed with and without a substimulatory dose of forskolin. Leucine alone (light bar) and leucine with forskolin (0.01 μM-heavy bar) were perfused through the vascular bed for a period of 30 minutes. Each point represent the mean ± SEM of three determinations.

4. DISCUSSION

The ability of neutral amino acids such as leucine to stimulate the transmural intestinal transport of dibasic amino acids is well documented. Lawless et al. (1987) proposed that leucine may interacting allosterically with the basolateral lysine carrier to increase its affinity for lysine and in this way accelerate the efflux from the enterocyte. The possible involvement of second messengers in this response was suggested because of the variability observed when attempting to reconstitute the system in BLMVs. It was hypothesized that perhaps leucine binds to some form of receptor, initiating a second messenger cascade which then modulates the activity of the intestinal basolateral transporter.

To investigate this possibility, at a preliminary level, I made use of the excellent *in vivo* preparation used by Cheeseman (1983) to investigate lysine transport across the intestinal basolateral membrane. The vascularly perfused frog preparation allows manipulation of the solution bathing either the mucosal side of the enterocyte or the serosal side. This then allows pharmacologic manipulation of second messenger systems. The approach taken to evaluate the role of second messengers in the phenomenon of leucine-stimulated lysine transport was initially a pharmacologic one. Various compounds exist which modulate the metabolism of second messenger systems and thus allow some insight into what role

these respective systems might play in a particular response (see Figures 1, 2). Information gained from such studies, alone, is not sufficient to ascribe a particular second messenger system to a physiological condition. Demonstration of elevation of the second messenger in question in response to a particular agonist must be achieved. As well, the response must take place when the second messenger system is stimulated via means other than by use of the native agonist. In this study, I have attempted to examine these criteria with respect to the leucine stimulation of lysine transport.

Evidence collected, to date, suggests that cAMP plays some role in regulation of intestinal transmural lysine transport. This statement is based on two lines of evidence, the first of which is inhibition of cyclic nucleotide catabolism. This is achieved with the aid of the phosphodiesterase inhibitor 3-isobutyl-1-methyl-xanthine (IBMX). Perfusing IBMX through the intestinal lumen (Figures 10, 11) while simultaneously perfusing leucine through the vascular bed markedly potentiates the response of lysine transport to leucine. This is what would be expected if leucine is stimulating the production of a cyclic nucleotide. Indeed, at three different doses of leucine, IBMX very markedly shifted the lysine-response curve to the left (Figure 12) indicating that cAMP may be involved in the leucine stimulation of lysine transport. However, if IBMX is inhibiting the breakdown of a cyclic

nucleotide such as cAMP why does it not stimulate the transport of lysine when perfused in the absence of leucine? Presumably IBMX would still alter the basal levels of cyclic nucleotides in the tissue in the absence of leucine. Perhaps the basal rate of cAMP production in enterocytes is so low that in the period of time allotted for IBMX perfusion in the absence of leucine the accumulation of cAMP is not great enough to have an effect on the lysine transporter. Then, when leucine is present, the rate of cAMP generation increases to a level where the presence of IBMX has a marked effect on cAMP levels in the tissue.

Further support of the hypothesis that cAMP is involved in the regulation of dibasic amino acid transport comes from the experiments designed to elevate cAMP levels in the enterocytes. This was achieved using dibutyryl cAMP (DbcAMP) and forskolin. Both compounds exhibited the ability to stimulate transmural lysine transport. The response to DbcAMP was not identical to stimulation with leucine (Figure 13). The onset of the response requires more time and the response does not begin to turn off until some time after the perfusate is switched to one without DbcAMP. This may represent the fact that we are relying on diffusion of DbcAMP to access the intracellular compartment and that this compound is resistant to hydrolysis (Posternak et al., 1962).

The experiments with forskolin exhibited properties which more closely approximate those seen when transmural lysine

transport is stimulated by leucine (Figure 14). The onset of stimulation is fairly rapid and begins to shut off when the forskolin is withdrawn. It is interesting to examine the dose-response curve to forskolin (Figure 15). While it is evident that the response of lysine transport to forskolin is dose-dependent, it is apparent upon closer examination that the response is rather flat. The forskolin concentration was increased through four orders of magnitude (i.e. 0.01 to 10 μM) yet the stimulation of transport only ranged from 1- to 2.3- fold. This suggests that perhaps the mechanism of forskolin stimulation of lysine transport is not identical to that caused by leucine which seems capable of stimulating the vascular appearance of lysine much more effectively.

The action of forskolin on the vascular appearance of lysine could be achieved by a number of different mechanisms, including the following: forskolin could be stimulating a diffusional component of lysine transport rather than stimulating the lysine transporter in the basolateral membrane. To dismiss this possibility, an important control experiment was conducted (Figure 16). The basis for these experiments is as follows: if the stimulation of transmural lysine transport by forskolin is due to an increased transport rate across the intestine, then when the preparation is exposed to a high concentration of a competing amino acid such as arginine (Cheeseman, 1983), the stimulation should be reduced or abolished.

This is because arginine will compete with lysine for transport. If the stimulation of lysine transport by forskolin is due to stimulation of a non-specific step, then the arginine should not have any effect on the increased appearance of radioactive lysine in the vascular perfusate. If, on the other hand, stimulation of lysine transport is achieved by some action on the dibasic amino acid transport mechanism, then the presence of arginine should reduce or abolish this stimulation. It seems that at least 80 % of the lysine vascular appearance is mediated by specific transport processes since the presence of 50 mM arginine can reduce transport to 20 % of its original value (Figure 16). As was demonstrated, forskolin had no effect on the appearance of radioactive lysine when competing amounts of arginine were present. This suggests that the mechanism of forskolin stimulation of lysine vascular appearance is via an action on the transport process itself.

There are several reports in the literature which clearly indicate that not all of the actions of forskolin are mediated by activation of adenylate cyclase and thus, cAMP (Coombs and Thompson, 1987; Hoshi et al., 1988; Perozo and Benzanilla, 1988; Wagoner and Pallotta, 1988). Hoshi et al. (1988) recorded inhibition of voltage-gated K^+ channels in PC12 cells. Both Perozo and Bezanilla (1988) and Coombs and Thompson (1987) observed similar effects on the outward K^+ currents in squid axons and nudibranch neurons, respectively. Wagoner and Pallotta (1988) noted that

forskolin accelerated the time-course of nicotinic acetylcholine receptor desensitization and this was not mediated by cAMP. Also, van-Valen and Keck (1988) noted that forskolin inhibited glucose transport in bone cell cu cultures via a cAMP-independent mechanism. therefore, important to ascertain whether or not the action of forskolin on lysine transport was due to the production of a second messenger like cAMP or was the result of a non-specific interaction of forskolin with some cell regulatory component. The experiments conducted with the forskolin derivative 1,9-dideoxy forskolin (DDF) go a long way to help address this question. All the non-cAMP mediated effects of forskolin reported (Coombs and Thompson, 1987; Hoshi et al., 1988; Perozo and Bezanilla, 1988; Wagoner and Pallotta, 1988; van-Valen and Keck, 1988)) could be reproduced by DDF yet not by addition of exogenous cAMP. Thus in my system, in which DDF was incapable of stimulating the vascular appearance of lysine (Figure 17a, 17b), it seems reasonable to assume that the effect of forskolin (i.e. stimulating lysine transport) was a cyclic nucleotide-dependent effect.

It is tempting to assume that if forskolin has an effect on a physiological process, that it must be due to cAMP. As I have just noted, this may not necessarily be the case. Likewise, however, if DDF is incapable of effecting the response, it does not mean that cAMP is responsible. Recently, it has been reported that forskolin is also capable of stimulating the production of cGMP (Ho et al.,

1989). Ho et al. (1989) observed that forskolin was capable of elevating cAMP and cGMP levels in dispersed rat pinealocytes in a dose-dependent manner. Caution is urged when they state, that based on these findings, it seems prudent to suspect that any effect of forskolin treatment may in part reflect an action of cGMP and that the forskolin = cAMP equation must be used cautiously.

Nevertheless, the results I have obtained, taken in context with the fact that forskolin does not seem to stimulate a passive component of lysine flux, suggests that the stimulation of transmural lysine transport is achieved by an action of a cyclic nucleotide on the transport process itself.

These data suggest two possibilities with regard to the mode of action of leucine and cyclic nucleotides on the transmural transport of lysine across the intestine. It is possible that leucine is binding to a membrane receptor on the enterocyte and this receptor stimulates the production of a cyclic nucleotide (i.e. cAMP, see Figure 22). Cyclic AMP, then, is the second messenger which changes the activity of the basolateral membrane transporter. It may achieve this, for instance, by phosphorylation of the transporter or a regulatory component which perhaps increases the affinity of the transporter for lysine.

Alternatively, cAMP production may not be stimulated by leucine. The experiments discussed to this point do not demonstrate that leucine is stimulating the production of

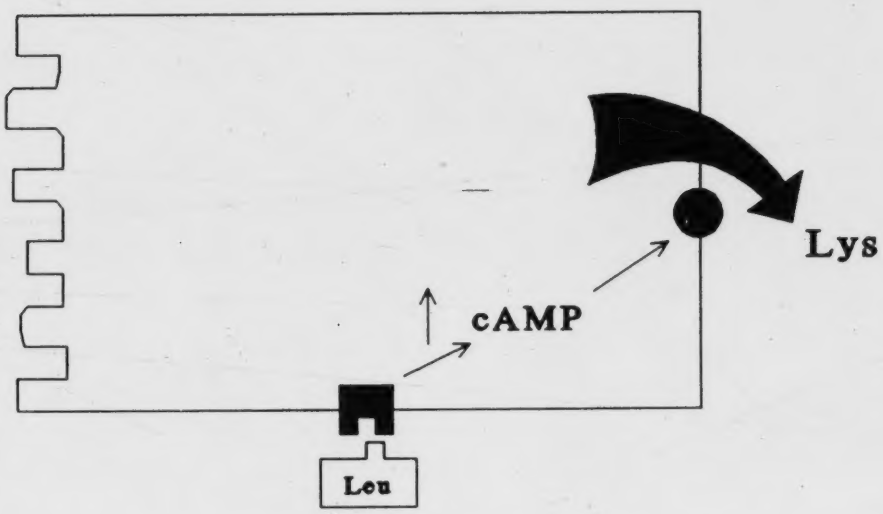

Figure 22. Possible method of stimulation of lysine transport by leucine. Leucine may bind to a receptor on the cell membrane and stimulate the production of cAMP. Cyclic AMP, then, through a protein kinase may alter the activity of the lysine transporter in the basolateral membrane, resulting in a much greater flux of lysine across this membrane.

cAMP. All I have shown is that a cyclic nucleotide can modify and enhance the transmural transport of lysine (i.e. forskolin and DbcAMP experiments). The experiments with IBMX, instead of necessitating the stimulation of cAMP production due to a leucine-receptor interaction could be explained as cAMP enhancing the responsiveness of the system to leucine. Yet, forskolin and DbcAMP can stimulate transport independently of the presence of leucine. This apparent disparity can be reconciled if we dedicate two roles for cAMP in this system. cAMP may be able to enhance the stimulatory action of leucine while at the same time is capable of promoting lysine absorption via some alternate or analogous mechanism (see Figure 23).

In order to discriminate between these two possibilities it is important to assay the levels of cyclic nucleotides in intestinal tissue under control conditions and in the presence of leucine.

Experiments of this nature were attempted with a number of preparations. Initially, I chose to attempt these experiments with rings of frog intestine. This preparation was chosen because the experiments were relatively easy to conduct and the tissue is likely to remain viable during the course of the experiment. As previously mentioned, it does not appear that leucine was capable of stimulating the production of cAMP or cGMP in this preparation (Table 1) while it is apparent that the presence of IBMX does result in a markedly elevated level of cAMP in the tissue. These

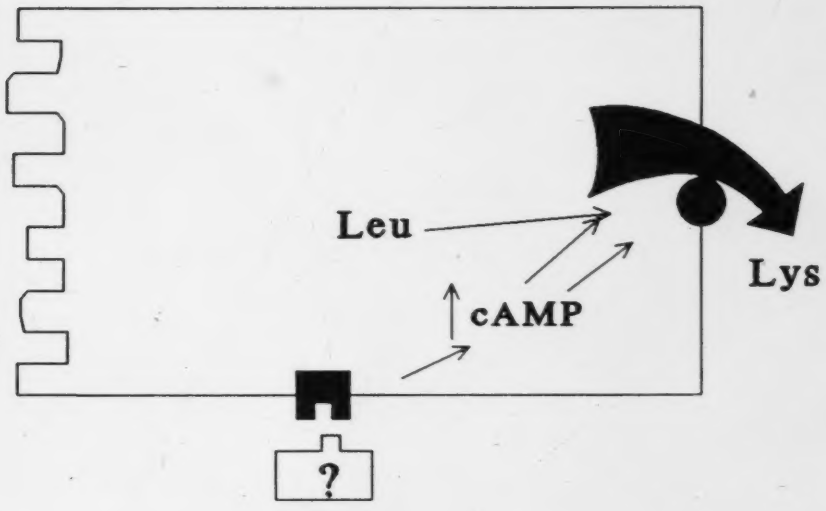

Figure 23. Possible mechanism of stimulation of lysine transport by leucine. Leucine may act to increase the activity of the basolateral lysine transporter independent of cAMP. An increase in cAMP via some unknown mechanism may enhance the responsiveness of the lysine transporter to leucine yet is capable of stimulating the lysine transporter directly.

results were considered inconclusive, however, because this preparation, upon closer consideration, revealed several potential problems. First, the rings of tissue were incubated in frog Ringers that was gassed, but this does not mean that the tissues were adequately oxygenated. The rings are relatively thick and this means that the oxygen must diffuse through the tissue to thoroughly oxygenate the entire ring. Secondly, when dispersing the rings it was necessary to use a sonicator and a great deal of effort was required to achieve satisfactory dispersal. As such, there was a fair amount of delay between thawing rings and achieving sufficient sonication. Therefore, any slight alterations in cyclic nucleotide levels may have been obliterated by the time the procedure was finished. A third consideration was that I was measuring total intestinal tissue cyclic nucleotide levels. Remember, the hypothesis calls for a localized increase of either cAMP or cGMP in the enterocyte specifically. I was measuring not only the cyclic nucleotide content of the enterocytes but of the various muscle layers, neurons, goblet cells, endocrine cells etc. In other words, I was looking for what are probably fairly minute changes in cyclic nucleotide concentrations in one cell type from a pool of many other cell types. Thus, if you like, the signal-to-noise ratio was probably very low, and might have obscured the slight changes necessary to support my hypothesis.

With these considerations in mind, I chose to attempt similar experiments on isolated enterocytes from rat jejunum. This preparation clearly minimizes the problem with the signal-to-noise ratio. Such an isolated cell preparation also allows direct exposure of the enterocytes to the drugs or amino acids in the test solution, unlike the previous experiment with rings where I was relying upon diffusion of compounds like IBMX to access the cytoplasm of the enterocyte. This preparation, like the previous, demonstrated that the cAMP content of the cells does not change with exposure to leucine (Table 3). In my mind, however, there was still some question as to the validity of these results. The fact that I was attempting to correlate observations in the frog intestine with experiments in rat impressed upon me the necessity of demonstrating the effect of leucine on cyclic nucleotide levels in a suitable preparation from the frog intestine.

The experimental approach I then adopted was to prepare mucosal scrapings from frog intestine perfused through the vascular bed with normal vascular perfusate (i.e. control conditions), or vascular perfusate with either leucine (i.e. test conditions), or forskolin (i.e. positive control) (Table 4). Again, this approach demonstrated that leucine did not have any effect on the concentration of cyclic nucleotides in this tissue. However, the suitability of this approach may be called into question since perfusion with forskolin, while it did increase the average levels of

both cAMP and cGMP in the scrapings, did not result in a significant difference between control and test values ($p < 0.05$).

Three experimental approaches were taken in an attempt to evaluate the effect of leucine on the cyclic nucleotide content of enterocytes. All three approaches yielded, qualitatively similar results (i.e. no effect of leucine on cyclic nucleotide levels). I feel that these experiments, while revealing, are not conclusive and therefore, I have conducted more experiments in the vascularly perfused frog in order to either lend support or contradict these findings.

A family of isoquinolinesulfonamide derivatives (H7, H-8, H-9, and HA-1004) and napthalenesulfonamide derivatives (ML-7, and ML-9) are protein kinase inhibitors. Such compounds are very useful in determining if a particular physiological response is mediated by a phosphorylating enzyme. The hypothesis that has been developed postulates that cAMP production may be triggered in the enterocytes by some action of leucine. It follows, then, that if this is the mode of action of leucine that cAMP-dependent protein kinase (PKA) would be involved in the response downstream of the production of cAMP (see Figure 1). It should, therefore, be possible to inhibit PKA with one of these compounds to determine if PKA is involved in this response; if the stimulation of lysine transport by leucine is

inhibited by HA-1004 it implies therefore that cAMP is involved.

There is, however, a slight problem with the family of inhibitors just mentioned, and that is that they are rather non-specific. All four of the isoquinolinesulfonamide derivatives tend to inhibit to a greater or lesser extent PKA, cGMP-dependent protein kinase (PKG) and protein kinase C (PKC). This makes interpretation of results rather speculative, but for the purposes of my investigation provides very useful information, regardless. I chose to use the derivative HA-1004 because it has a relatively low K_i for PKA while, of the four derivativesm, it has the highest K_i for PKC.

As demonstrated, HA-1004 was unable to inhibit the effect of leucine on transmural lysine transport (Figure 18). Similar observations were noted in initial experiments using H-7, a compound most specific for PKC (Figure 19). Contrary to what might have been expected, both HA-1004 and H-7 actually stimulated the response of lysine transport to leucine. This finding suggests several possibilities. First and foremost, it would appear that PKA is not directly involved in the leucine stimulatory phenomenon. If it was, the stimulatory effect of leucine would have been expected to be reduced or absent in the presence of HA-1004. This, by inference, lends support to the idea that leucine does not directly stimulate the production of cAMP or for that

matter, cGMP (since the K_i for PKG is almost the same as for PKA).

The stimulation of the leucine response is an interesting finding. It may imply one of several possibilities in the regulation of intestinal transmural lysine transport . It may be that the second messenger involvement in this response is more intricate that first imagined. If leucine is stimulating lysine transport via some kind of second messenger system, it may be that inhibition of a protein kinase (i.e. PKA, PKG, PKC) is a requisite part of achieving optimal stimulation of this second messenger system. For example, leucine may be acting on two or more receptors that induce a cooperative effect between the second messengers which would be required to produce a full response to leucine. Such phenomena are well documented. The work of Klein and his colleagues amply demonstrates this sort of dual receptor regulation of cAMP or cGMP production in rat pinealocytes (Klein et al., 1983; Vanacek et al., 1985; Klein et al., 1988). They have determined that in order to obtain a similar level of response in pinealocytes (in terms of cAMP or cGMP production) as obtained by epinephrine, stimulation of both α_1 and β receptors must occur.

Alternatively, one could imagine a scenario of stimulatory and inhibitory pathways converging on the lysine transporter that must be acted upon in unison to obtain maximal stimulation. As an example, perhaps by inhibiting the activity of a protein kinase with HA-1004 or H-7 we are

inhibiting a process normally going on in the basal state of the tissue which reduces the activity of the basolateral membrane lysine transporter. Leucine may act on both a direct stimulatory component and a inhibitory component regulating the lysine transporter. Thus, by inhibiting the action of a specific protein kinase we may be releasing some sort of tonic inhibition of the lysine transporter which may allow it to be more responsive to the stimulatory pathway.

Clearly, the two hypotheses put forward are from a number of possibilities that may be suggested to explain the data. However, I feel that not too much emphasis should be placed on the experiments with the isosquinolinesulfonamide derivatives because it must be remembered that these experiments are only preliminary and far from conclusive. For example, I have not demonstrated that HA-1004 and H-7 are directly inhibiting the activity of enterocyte protein kinases. This clearly must be confirmed in order to draw a correlation between cause and effect. These results do, however, prove intriguing and may suggest which further avenues of research may prove fruitful.

The role of cyclic nucleotides in lysine transport is becoming somewhat clearer. Evidence from the cyclic nucleotide assays and the protein kinase inhibition studies all suggest that the role of cyclic nucleotides is not direct (i.e. leucine does not directly stimulate, for instance, cAMP production. How then are the experiments with IBMX explained? Is it possible that inhibition of

cyclic nucleotide metabolism, is capable of elevating basal cyclic nucleotide levels to a level which can modulate (enhance) the response of the lysine transporter to leucine without affecting transport of lysine in the absence of leucine? In an effort to test this possibility I conducted experiments with substimulatory levels of forskolin, as determined by the dose-response curve (Figure 15). This was an attempt to mimic the conditions set up in the IBMX perfusion experiments. I assumed that a substimulatory dose of forskolin (i.e. 0.01 μM) although unable to stimulate lysine transport on its own, would have an affect on the basal levels of cyclic nucleotide (i.e. cAMP) in the enterocytes. This then would mimic the conditions that may exist with IBMX (i.e. elevation of basal cAMP levels to a point not great enough to alter transport on its own). As shown in Figure 21, these conditions can enhance the leucine stimulation of lysine transport just as IBMX did and thus lend credence to the hypothesis that IBMX and forskolin can enhance the leucine stimulation of lysine transport by modulating the response to leucine via a increased levels of basal cyclic nucleotides.

4.1. Summary

Throughout this thesis I have cited numerous experiments that I have conducted which argue either for, or against the involvement of cAMP in the stimulation of transmural lysine

transport by leucine. I would like to now summarize these findings in point form:

Experiments supporting a direct role for cAMP in the stimulation of lysine transport by leucine.

1. IBMX potentiates the response of lysine transport to stimulation by leucine and shifts the entire lysine response curve to leucine to the left.

2. Transmural lysine transport can be stimulated by exogenous cAMP (DbcAMP) and by forskolin.

3. Forskolin has no effect on transmural lysine transport when lysine is perfused through the intestinal lumen in the presence of competing amounts of arginine.

4. 1,9-dideoxy forskolin has no effect on transmural lysine transport.

Experiments supporting an indirect role for cAMP in the stimulation of lysine transport by leucine.

1. Cyclic AMP concentrations in various preparations of both frog and rat intestine were not altered by exposing the tissues to leucine.

2. The protein kinase inhibitors, HA-1004 and H-7, did not inhibit the stimulation of lysine transport by leucine.

3. Forskolin at a sub-stimulatory dose potentiated the stimulation of lysine transport by leucine in a manner similar to that observed with IBMX.

4.2. Future research - directions

This work presents information that can be taken in several directions. The potential for investigating the leucine effect in a cell culture line exists. This, of course, would be dependent upon demonstrating that leucine is capable of stimulating lysine transport in the cell line. Such investigations would enable accurate delineation of the second messenger involvement in such a phenomenon. But I urge caution when applying the results of such work to the situation occuring *in vivo*. It must be remembered that cell lines are composed of transformed cells which, in all probability, do not act like the normal cells they are supposed to represent.

The vascular perfused frog preparation will prove invaluable in further investigations. The role of the inositol lipid second messenger pathway, not thoroughly examined in this work can be investigated with this preparation. Compound such as phorbol esters, Ca^{2+} channel blockers such as lanthanum, and Ca^{2+}/calmodulin inhibitors

could all be used to examine the role of this pathway more completely.

A potentially very informative study would be to investigate the state of phosphorylation of basolateral membrane proteins. Many second messenger pathways culminate in the phosphorylation of a membrane protein. I think it would be worthwhile to determine if, in response to leucine there is a phosphorylation step occuring. One could potentially examine this using gel electrophoresis or in the vascularly perfused frog small intestine. Sodium azide inhibits phosphatase activity (Dawson and Cook, 1988). As a result, after stimulating transport with leucine perfused with an appropriate concentration of sodium azide, the rate at which lysine transport returns to basal levels should be delayed if phosphorylation of a protein is involved. This would certainly lend support to the idea of second messenger involvement in this phenomenon.

The potential for productive research work in this area is limitless. I have only touched on a few of the many possible areas which will provide interesting findings. Clearly, many more strategies of investigation will reveal themselves as this research is pursued.

5. CONCLUSION

The modulation of the intestinal, enterocyte basolateral membrane lysine transporter by leucine does not appear to involve, directly the production of a cyclic nucleotide. cAMP appears to be able to modulate (enhance) the reactiveness of this carrier to leucine. Stimulation of the carrier could also be achieved directly via cAMP generation; however, the magnitude of response seen with leucine could not be obtained. The involvement of other second messenger systems has not been adequately addressed. The role of Ca^{2+}/ calmodulin and closer investigation of the role of protein kinases merits examination. Some potential role for protein kinases seems plausible. A direct allosteric modulation of the basolateral lysine carrier by leucine as originally proposed by Lawless et al. (1987) cannot yet be ruled out. Indeed, it may be that certain second messenger systems serve only to alter the responsiveness of the system to leucine.

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